THE UNIVERSITY of EDINBURGH

Title	Effects of alteration of the dietary amino acid balance on brain neurotransmitter concentrations and patterns of growth and food intake in the chick
Author	Harrison, Lydia Margaret.
Qualification	PhD
Year	1987

EFFECTS OF ALTERATION OF THE DIETARY AMINO ACID BALANCE ON BRAIN NEUROTRANSMITTER CONCENTRATIONS AND PATTERNS OF GROWTH AND FOOD INTAKE IN THE CHICK

LYDIA MARGARET HARRISON

DOCTOR OF PHILOSOPHY
UNIVERSITY OF EDINBURGH
1987

The work contained in this thesis is my own original work and has not been submitted for a degree at any other University.

L. Harrison.

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ACKNOWLEDGEMENTS

I wish to thank past and present members of the Agricultural Biochemistry Department of The Edinburgh School of Agriculture for their support and encouragement during the course of this work. Particular thanks are due to Dr. T. Acamovic for much advice on the methodology and problems of HPLC, and to Mr. G. Walker for many hours of invaluable assistance during the feeding experiments.

My thanks are also due to Dr. J.P.F. D'Mello for his continual advice and encouragement during the course of this work, and for his constructive criticism of this thesis.

I am grateful to the staff of the Central Analytical Laboratory of the School of Agriculture for the proximate analysis of all experimental diets and the determination of the dietary zinc content where required.

I am very grateful to Miss P. Phillips for much statistical advice.

Thanks are also due to all those who provided invitations to dinner and much encouragement in the completion of the work described and writing of this thesis.

This work was funded by a Research Assistantship from The Agricultural and Food Research Council, for which I am very grateful.

Abbreviations used in this thesis

AADC	aromatic L-amino acid decarboxylase
ADH	aldehyde dehydrogenase
AME	apparent metabolisable energy
AME(N)	apparent metabolisable energy corrected for dietary nitrogen content
ATP	adenosine triphosphate
BCAA	branched-chain amino acid(s)
COMT	catechol-o-methyltransferase
DA	dopamine
DOPA	dihydroxyphenylalanine
DOPAC	dihydroxyphenylacetic acid
E	epinephrine
ECD	electrochemical detection
EDTA	ethylenediaminetetraacetic acid (disodium salt)
GC	gas chromatography
HIOMT	hydroxyindole-o-methyltransferase
HPLC	high-performance liquid chromatography
5HIAA	5-hydroxyindoleacetic acid
5HT	5-hydroxytryptamine
5HTP	5-hydroxytryptophan
HVA	homovanillic acid
k'	phase capacity factor
LNAA	large neutral amino acid(s)
MAO	monoamine oxidase
NAT	N-acetyltransferase
NE	norepinephrine
NEFA	non-esterified fatty acids
OPA	ortho-phthalaldehyde
pCPA	parachlorophenylalanine
PFPA	pentafluoropropionic anhydride
RIA	radioimmunoassay
TLC	thin-layer chromatography
VMH	ventromedial hypothalamus

Amino acid abbreviations follow the recommendations of the I.U.P.A.C-I.U.B. Commission on Biochemical Nomenclature. Other abbreviations assume the normal chemical and I.U.P.A.C. conventions.

ABSTRACT

High-performance liquid chromatography (HPLC) was employed for the simultaneous determination of the catecholamines norepinephrine (NE), epinephrine (E) and dopamine (DA), the DA metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); and the indoleamine 5-hydroxytryptamine (5HT) and its metabolite 5-hydroxyindoleacetic acid (5HIAA) in the chick brain.

The effects of classical amino acid antagonisms and imbalances and other variations in the amino acid balance of the diet, on the patterns of growth and food intake of the chick and on its brain concentrations of the above compounds, was studied. Under appropriate conditions the chick brain concentrations of NE, DA and 5HT were affected by variations in the dietary content of their indispensable amino acid precursors.

The intake of a diet which was imbalanced with respect to tyrosine+phenylalanine was increased by reduction of the dietary zinc content. This coincided with a reduction in the brain concentration of NE, but there was no correlation of the concentration of this or any other catecholamine in the chick brain with the quantity of food consumed.

The existence of a functional blood-brain barrier in the young chick is suggested, particularly by the reduction of catecholamine and 5HT concentrations in the brains of chicks receiving diets containing excessive amounts of the branched-chain amino acids (BCAA) proposed to compete with the neurotransmitter precursors for brain uptake. However, the accompanying decrease in intake of food and hence neurotransmitter

precursors, of birds fed diets containing the excessive BCAA cannot be completely ruled out as a contributing factor.

Changes in the growth and food intake of birds fed diets which are distorted in their amino acid content are not accompanied by consistent alterations in the brain concentrations of any of the neurotransmitters measured. Some correlations are observed between the concentrations of NE, DA or 5HT and the quantities of food or protein ingested, or the intake of apparent metabolisable energy corrected for the dietary nitrogen content (AME(N)). However, most of these coincide with correlations of neurotransmitter concentrations with the quantity of the relevant precursor consumed.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

The performance of animals as measured by their growth and food intake is dependent to a very large extent on the nutritional quality of the diet consumed. For optimal performance to be attainable, this diet must provide the animal with a supply of all the mineral compounds and vitamins which it requires, sufficient energy for its activities and adequate sources of protein. Dietary protein constitutes both a supply of amino acids for synthesis of the animal's own tissue proteins and a source of nitrogen in the production of biologically active amines and amino acid derivatives. Amino acids which may be synthesised by the animal from the carbon and nitrogen sources in the diet are termed 'dispensable' amino acids, while those which cannot be synthesised within the body but are required, are termed 'indispensable'. A diet must therefore supply indispensable amino acids in amounts adequate for the animal's needs, together with a quantity of dispensable amino acids sufficient for its other synthetic requirements, these including production of any of the dispensable amino acids not adequately present within the diet itself.

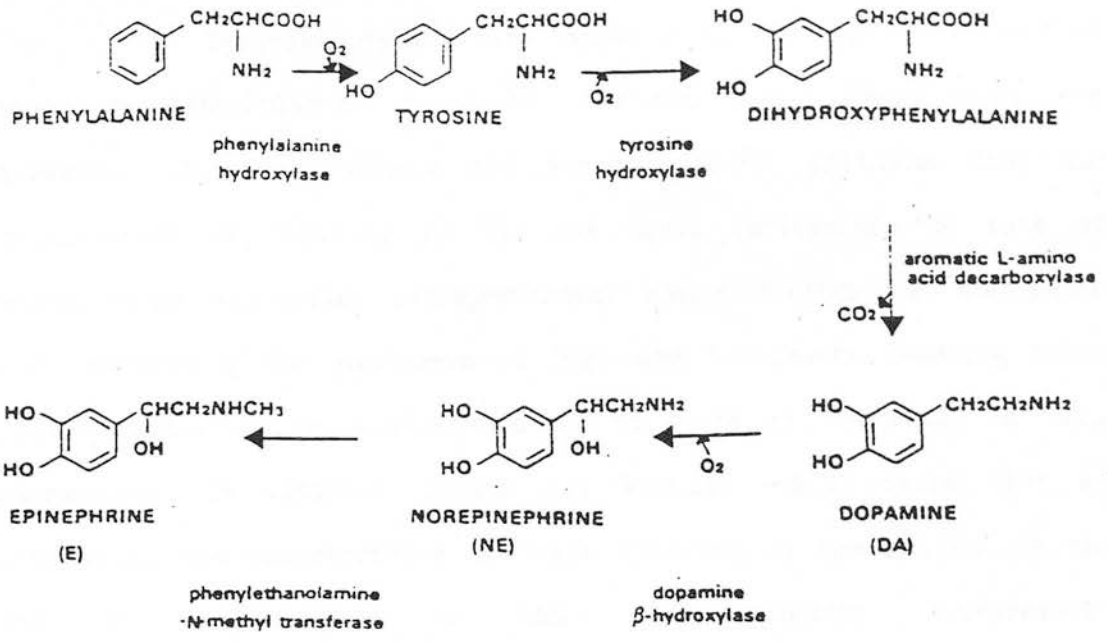
The studies to be described were designed to investigate the effects of variations in the dietary content and balance of indispensable amino acids, on the patterns of food intake and weight gain of young broiler chicks and the concentrations in their brains of certain amines derived from indispensable amino acids and acting as neurotransmitters in the central nervous system. Information currently available in these areas will be reviewed, but initially the neurotransmitters in question and aspects of their synthesis and degradation must be considered.

1.1 Neurotransmitter biosynthesis

The neurotransmitters to be studied are derived from indispensable amino acids, whose only sources therefore are the proteins and amino acids ingested. The catecholamines norepinephrine (noradrenaline, NE), epinephrine (adrenaline, E) and dopamine (3-hydroxytyramine, DA) are derived from tyrosine which is available in the diet or produced from the indispensable amino acid phenylalanine; and the indoleamine 5-hydroxytryptamine (serotonin, 5HT) is derived from tryptophan. The biosynthetic pathways for these neurotransmitters are shown in Fig. 1.

a. Catecholamine biosynthesis

L-tyrosine is actively taken up into the the nerve terminals of noradrenergic and dopaminergic neurones and converted by tyrosine hydroxylase (E.C. 1.14.6.2) in the cytoplasm to L-3,4-dihydroxy-phenylalanine (DOPA). This enzyme also catalyses the hydroxylation of phenylalanine to tyrosine, at high concentrations of phenylalanine, formation of DOPA being inhibited and the greater proportion of the product being tyrosine (Ikeda, Levitt and Udenfriend, 1967). Removal of CO_2 from DOPA is by aromatic L-amino acid decarboxylase (AADC, E.C. 4.1.1.28), often termed DOPA decarboxylase, the product being DA, which is stored in the synaptic vesicles of dopaminergic neurones, complexed with proteins, divalent cations and ATP (Kruk and Pycock, 1979). In noradrenergic nerve endings, DA is transported actively into storage vesicles and converted enzymatically by dopamine β-hydroxylase (E.C. 1.14.17.1) to NE by oxidation of the side chain. In some neurones in the CNS, NE is methylated to E by phenylethanolamine-N-methyltransferase (E.C. 2.1.1.28).



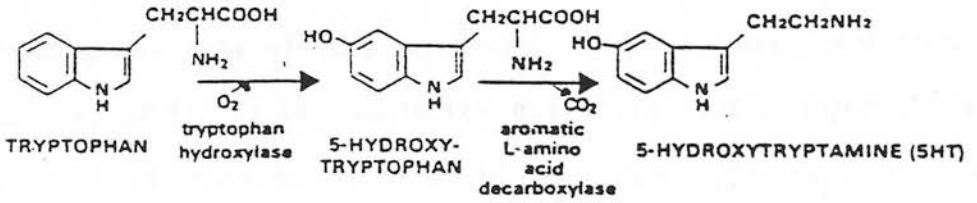


Fig. 1. Biosynthesis of the catecholamines and 5-hydroxytryptamine

b. Regulation of catecholamine biosynthesis

Tyrosine hydroxylase appears to be the rate-limiting enzyme in catecholamine synthesis. Wurtman, Larin, Mostafapour and Fernstrom (1974) and Gibson and Wurtman (1977) indicated that the concentration of tyrosine in the rat brain influenced the rate of catecholamine synthesis, intraperitoneal administration of the amino acid accelerating the synthesis of DOPA and treatments lowering brain tyrosine resulting in a reduction in the rate of synthesis of this intermediate. In addition, Gibson and Wurtman (1978) found that an increase in the concentration of brain tyrosine in the rat raised the rate of accumulation of the NE metabolite 3-methoxy,4-hydroxyphenylglycol. These data indicated that the brain concentrations of the catecholamines might be dependent on the availability of their amino acid precursor. However, the importance of brain concentrations of tyrosine and phenylalanine in the regulation of catecholamine synthesis is not entirely clear. A low intraperitoneal dose of phenylalanine was found to elevate the rat brain concentration of tyrosine without increasing the rate of DOPA synthesis (Wurtman, Larin, Mostafapour and Fernstrom, 1974), while Westerink and Wirix (1983) reported that the brain pool of tyrosine appeared to be large. Tyrosine hydroxylase is also reported to be inhibited by the end-products DA and NE (Kruk and Pycock, 1979).

c. Biosynthesis of 5-hydroxytryptamine

In the cytoplasm of serotinergic neurones, tryptophan hydroxylase (tryptophan 5-monooxygenase, E.C. 1.14.16.4) acts on L-tryptophan to form 5-hydroxytryptophan (5HTP), which is decarboxylated to 5HT by AADC (also termed 5HTP decarboxylase). The neurotransmitter is actively transported into storage vesicles where it is believed to be

complexed in a manner similar to that of NE and DA (Kruk and Pycock, 1979).

d. Regulation of 5HT biosynthesis

Tryptophan hydroxylase is the rate-limiting step in 5HT synthesis (Friedman, Kappelman and Kaufman, 1972), being thought to have a k_m of approximately 50μM both *in vitro* (Kaufman, 1974) and *in vivo* (Sved, 1983), although recent work suggests that this value may be somewhat high (Wolf and Kuhn, 1986). The enzyme appears to be unsaturated at physiological concentrations of tryptophan, and an increase in the brain concentration of this amino acid is able to increase the rate of synthesis of 5HT (Carlsson and Lindqvist, 1972). Macon, Sokoloff and Glowinski (1971) demonstrated that end-product inhibition of 5HT synthesis might occur when 5HT concentrations in the brain were increased in the presence of inhibitors of its breakdown, and this was confirmed by Hamon, Bourgoin and Glowinski (1973), studying incubations of rat brain slices. This end-product inhibition of 5HT synthesis may not however actually operate within physiological concentrations of 5HT (Fernstrom, 1983). Nevertheless, it appears that 5HT production does not continually increase with increasing administration of tryptophan. Young and Gauthier (1981) reported that patients receiving 3g of this amino acid orally showed an increase in its plasma concentration and an increase in the cerebrospinal fluid concentrations of both tryptophan and 5HIAA. However, while doubling the dose raised the plasma tryptophan concentration further, no additional rise in the cerebrospinal fluid concentrations of tryptophan or 5HIAA was observable.

e. Requirement for minerals and vitamins in catecholamine and 5HT biosynthesis .

i. Minerals

Metal ions are cofactors of several of the enzymes functioning in catecholamine and 5HT metabolism, these being of magnesium for COMT, copper for MAO and dopamine-β-hydroxylase and iron for enzymes hydroxylating tyrosine, phenylalanine or tryptophan (Sourkes, 1972; Youdim, Hamon and Bourgoin, 1975; White, Handler, Smith, Hill and Lehman, 1978). Copper-deficient rats have been shown to have lowered brain concentrations of the catecholamines and a reduced brain activity of tyrosine hydroxylase (Morgan and O'Dell, 1977). In addition, Sourkes (1972) reported that the conversion of DA to NE in rat heart tissue was reduced during copper deficiency. A deficiency of iron has been observed to reduce the activity of MAO in rat tissues (Youdim, Grahame-Smith and Woods, 1976) and also to alter the response of the post-synaptic neurone to 5HT and DA (Youdim and Green, 1978).

Some effect of zinc deficiency on brain catecholamine concentrations is also apparent. Recent work reported that zinc-deficient rats showed increased brain concentrations of NE (Wallwork, Botnen and Sandstead, 1982). It was suggested that this might be due to the possible requirement for zinc of the dehydrogenase enzyme which acts in conjunction with MAO for neurotransmitter breakdown. Reeves and O'Dell (1984) however, indicated that while zinc deficiency alone had no effect on concentrations of NE and DA in the anterior hypothalamus of the rat, reduction of the dietary tyrosine content caused a reduction in these concentrations in zinc-deficient rats.

ii. Vitamins

It is known that ascorbic acid (vitamin C) is involved in the reduction of the metal ions in the functioning of dopamine-β-hydroxylase, while the enzymes hydroxylating tyrosine, phenylalanine and tryptophan employ tetrahydrobiopterin, this prosthetic group being derived from folic acid (White *et al.*, 1978). The activity of aromatic-L-amino acid decarboxylase requires pyridoxal phosphate (vitamin B_6) (Lovenberg, Weissbach and Udenfriend, 1962) and that of MAO depends on riboflavin (vitamin B_2) as a precursor of its flavin nucleotide prosthetic group (White *et al.*, 1978). Little data are available on the effects of vitamin deficiencies on the synthesis of the catecholamines and 5HT, although pyridoxine-deficient rats have been shown to have reduced brain concentrations of 5HT, but not DA (Dakshinamurti, LeBlancq, Herchl and Havilicek, 1976).

f. Breakdown of catecholamines and 5HT

The degradation of NE, E, DA and 5HT is shown in fig. 2. The action of DA in the synaptic cleft is terminated by its active reuptake into the presynaptic nerve ending. Most of it is then taken again into the storage vesicles, any remaining neurotransmitter being destroyed enzymatically. Monoamine oxidase (MAO, E.C. 1.4.3.4.) is bound to the outer membranes of all mitochondria and in combination with aldehyde dehydrogenase deaminates DA oxidatively to 3,4-dihydroxyphenylacetic acid (DOPAC). Both DA and DOPAC are substrates for catechol-o-methyltransferase (COMT, E.C. 2.1.1.6), an enzyme which is both cytoplasmic and extracellular (Kruk and Pycock, 1979), the products being 3-methoxytyramine and homovanillic acid (HVA, 3-methoxy,4-hydroxyphenylacetic acid) respectively.

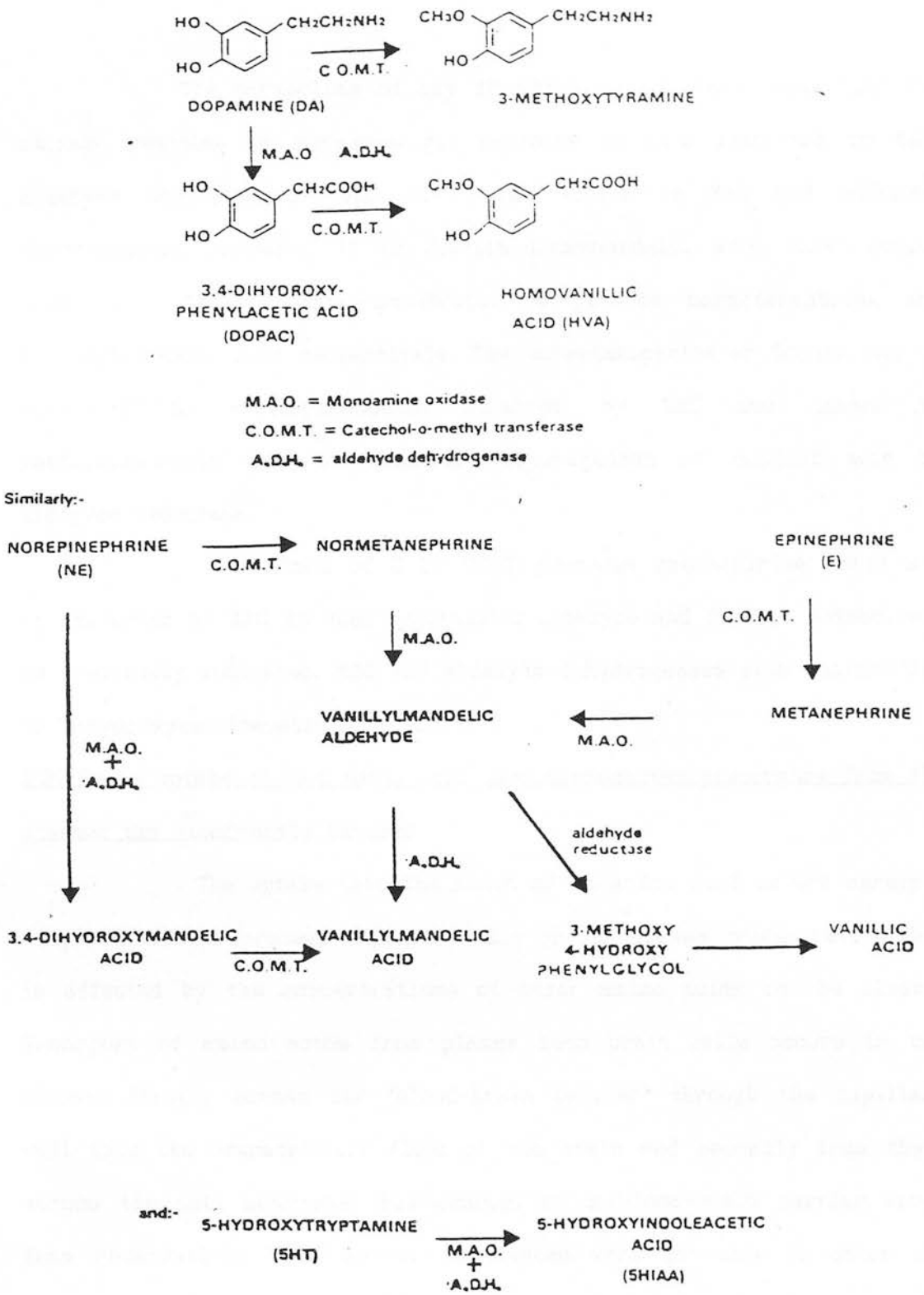


Fig. 2. Breakdown of the catecholamines and 5HT

The catabolism of any NE which is not taken back into the storage vesicles of noradrenergic neurones is also initiated by MAO, aldehyde dehydrogenase and COMT. The action of MAO and aldehyde dehydrogenase converts NE to 3,4-dihydroxymandelic acid, COMT acting upon NE and this metabolite to produce normetanephrine and vanillylmandelic acid respectively. The normetanephrine so formed may be converted to vanillylmandelic aldehyde by MAO and thence to vanillylmandelic acid by aldehyde dehydrogenase or vanillic acid by aldehyde reductase.

Breakdown of E by COMT produces metanephrine which may be converted by MAO to vanillylmandelic aldehyde and further metabolised as previously indicated. MAO and aldehyde dehydrogenase also convert 5HT to 5-hydroxyindoleacetic acid (5HIAA).

1.2. Brain uptake of the amino acid neurotransmitter precursors from the plasma: the blood-brain barrier

The uptake into the brain of an amino acid is not merely a simple diffusion process dependent only on its plasma concentration, but is affected by the concentrations of other amino acids in the plasma. Transport of amino acids from plasma into brain cells occurs in two stages; firstly across the 'blood-brain barrier' through the capillary wall into the extracellular fluid of the brain and secondly from there across the cell membrane. The concept of a blood-brain barrier arose from observations that certain substances were not able to enter the brain to any significant extent when injected intravenously. In mammals and birds, the brain capillaries are not highly permeable, the endothelial cells of the walls being tightly connected and the entry of large molecules strictly controlled. This barrier exists in all areas of the brain except the hypothalamus, substances being able to diffuse

readily from the blood into the extracellular fluid of this region. The absence of a barrier in this area is important, as the hypothalamus responds to changes in the blood concentrations of compounds such as glucose and electrolytes and is involved in the regulation of these concentrations (Benzo, 1983). The structure of the avian blood-brain barrier has been considered by Stewart and Wiley (1981).

a. Competition between amino acids for brain uptake

Uptake of amino acids at the blood-brain barrier has been studied extensively. Using radiolabelled amino acids and tritiated water, three main amino acid transport systems were found (Olendorf, 1971; Olendorf and Szabo,1976), one transporting the basic arginine, lysine and ornithine, another for aspartic and glutamic acids and a third for the neutral phenylalanine, leucine, tyrosine, isoleucine, methionine, tryptophan, valine, cysteine, histidine, threonine, glutamine, asparagine and serine, though affinity for the last four was low. It was found that the k_m for the first few neutral amino acids was close to their plasma concentrations (Pardridge and Olendorf, 1975). It was thus possible that competition between these amino acids for transport across the blood-brain barrier into the tissue could occur, that is, the uptake of one neutral amino acid could be affected by the relative concentrations of the other neutral amino acids in the plasma.

Inhibition of uptake of one amino acid into the brain by another had been shown as early as 1961 when Neame, incubating rat brain slices with various groups of amino acids, showed that phenylalanine inhibited the transport of certain amino acids but particularly tyrosine into the brain. Later workers indicated that tryptophan and phenylalanine were mutually inhibitory of their uptake into brain slices (Barbosa, Herreros and Ojeda 1971), while Tews, Good

and Harper (1978) were similarly able to show that the uptake of tryptophan was most strongly inhibited by phenylalanine or a mixture of the branched-chain amino acids leucine, isoleucine and valine together with methionine.

In vivo experimentation showed that the intraperitoneal or intravenous administration of excessive amounts of an amino acid could affect the brain concentrations of others. Administration of a large dose of phenylalanine was shown to deplete the brain of valine, histidine, leucine, isoleucine and arginine in the rat, leucine administration in turn causing reductions of valine, phenylalanine and arginine concentrations in the brain (Roberts, 1968). McKean, Boggs and Peterson (1968) reported that high levels of phenylalanine caused a fall in the brain concentrations of most indispensable amino acids, while Banos, Daniel and Pratt (1974) showed that arginine and lysine competitively inhibited uptake of each other into the brain *in vivo*. Recent work by Ablett, MacMillan, Sole, Toal and Anderson (1984) studied the effect of administration of tyrosine to the rat by intraperitoneal injection in the presence and absence of a mixture of the branched-chain amino acids leucine, isoleucine and valine. They reported that the presence of these amino acids reduced the uptake of tyrosine into the rat brain by 48%, but had no effect on the increase in tyrosine concentrations observed in the plasma and other measured tissues.

Most of the above demonstrations of reductions in the brain concentrations of certain amino acids by administration of others, whether *in vitro* or *in vivo*, are in accord with what would be expected as a result of competition for brain uptake between amino acids having a common transport system. The brain concentration of an amino acid is therefore affected both by its plasma concentration and by the plasma

concentrations of other amino acids competing with it for uptake across the blood-brain barrier. Pardridge (1984) stated that the area of the brain which was directly accessible to amino acids was trivial in proportion to that covered by the blood-brain barrier. Therefore while entry into the brain in the hypothalamic area occurs without competition, it appears that this does not in general produce an effect on brain amino acid concentrations sufficient to mask the competition for uptake occurring elsewhere.

b. Maturity of the blood-brain barrier

Studies carried out on young animals and birds indicate that the blood-brain barrier may not be fully developed at birth or hatching and that the facility with which certain substances are able to enter the brain decreases with increasing maturity. Early work using radioisotopes showed that the barrier to chloride ions in the rat and the chick increased with age (Lajtha, 1957). A similar phenomenon in the uptake of amino acids into the brain has also been reported. Levi and Morisi (1971) studied changes in the pattern of free amino acids in the brain of the chick during its development. They reported that a progressive decrease in the brain concentrations of the free amino acids was accompanied by a fall in the correlation of plasma amino acid concentrations with those of the brain. Similar data were provided by Purdy and Bondy (1976), it appearing that the barrier to dispensable amino acids such as proline developed to a greater extent after hatching than that to the indispensable amino acids.

Work on the young rat also provided evidence that the blood-brain barrier was not fully mature at birth. Ashley, Fleury, Hardwick, Leathwood and Moennoz (1984) studied the effects of oral doses of tryptophan in the presence and absence of a mixture of other large

neutral amino acids (LNAA), on brain concentrations of tryptophan in the rat at five, eleven and twenty-one days of age. The LNAA mixture was composed of valine, methionine, isoleucine, leucine, phenylalanine and tyrosine-those amino acids most likely to compete with tryptophan for uptake across the blood-brain barrier (Olendorf and Szabo, 1976). If the brain concentration of tryptophan had been greater when this amino acid was administered alone than when it was given in the presence of the LNAA, then the existence of competition for brain uptake across a functional blood-brain barrier would have been supported. However, rats receiving tryptophan alone or in combination with the LNAA showed no such difference in brain tryptophan concentrations. It was also reported that in five-day old rats the plasma concentration of tryptophan alone and the plasma ratio of tryptophan to the sum of the LNAA concentrations ratio correlated equally well with the brain concentration of tryptophan, while for those at twenty-one days of age, only the ratio was significantly correlated with the brain concentration of this amino acid. It thus appeared that the blood-brain barrier of the rat continued to develop with age and that the effects of competition between the amino acids for uptake into the brain became progressively more important.

In both the rat and the chick therefore, the brain concentration of an amino acid may be affected both by its concentration in the plasma and by the plasma concentrations of amino acids likely to compete with it for uptake into the brain, to a degree depending on the maturity of the animal and the extent to which amino acid entry into the brain might be restricted by the blood-brain barrier.

1.3 Effect of the diet on amino acid concentrations in plasma and brain

As the brain amino acid content may be affected by the plasma amino acid pattern, that is, the plasma concentrations of the amino acids in relation to each other, it follows that the feeding of diets which alter this pattern should affect brain amino acid concentrations.

In dietary investigations, Fernstrom and Wurtman (1972a) found that administration of insulin or a carbohydrate diet to previously fasting rats resulted in an increase in plasma tryptophan of 30-40%. Fernstrom, Larin and Wurtman (1973) showed that feeding a diet containing only carbohydrate and fat caused a rise in brain tryptophan in the rat that was prevented by additional protein consumption. It had actually been expected that the increased intake of tryptophan would have been reflected in a corresponding rise in the brain concentration of this amino acid. Instead it was observed that the brain concentration of tryptophan correlated well with the ratio of its plasma concentration to the plasma concentrations of tyrosine, phenylalanine, leucine, isoleucine and valine, either singly or in total, -these LNAA competing with tryptophan for uptake across the blood-brain barrier (Olendorf and Szabo, 1976). A carbohydrate diet, causing the release of insulin, resulted in an increase in plasma tryptophan, a fall in the levels of the other LNAA competing with it for uptake by the brain, and hence an increase in the uptake of tryptophan into the brain. The brain concentration of tryptophan therefore rose. In contrast, consumption of protein raised the total plasma concentration of competing LNAA more than it raised plasma tryptophan concentrations. Uptake of tryptophan and hence the brain concentration of this amino acid was therefore not increased.

A similar pattern of amino acid interactions which affected the brain concentration of tyrosine was observed by Fernstrom, Hirsch and Faller (1976). They reported that the brain concentration of this amino acid correlated well with the ratio of the serum concentration of tyrosine to the sum of the serum concentrations of the LNAA competing with it for uptake into the brain-these being tryptophan, phenylalanine leucine, isoleucine and valine. Further work by Fernstrom and Faller (1978), indicated that when rats were fed carbohydrate-fat diets the levels of tyrosine, tryptophan and phenylalanine in the brain rose while the branched-chain leucine, isoleucine and valine fell. Protein suppressed the rise in aromatic and attenuated the fall in branched-chain amino acids. Once again, the changes in brain amino acid levels correlated well with the ratio of their serum levels to the total LNAA concentration.

The above investigations thus provided evidence that the brain concentrations of tyrosine, phenylalanine and tryptophan, the precursors of the catecholamines and 5HT, were dependent on the ratio of their plasma concentrations to the total concentration of their competitors for brain uptake, rather than on their actual concentrations in the plasma. It was also apparent that manipulation of the diet could bring about changes in both the plasma amino acid pattern and brain amino acid concentrations.

1.4 Albumin binding of Tryptophan

Almost all amino acids are carried in the plasma in the free form. Tryptophan however, may be either free or bound to plasma albumin. Concurrently with the previously-described investigations of Fernstrom and co-workers, the extent to which the binding of tryptophan to albumin might also affect the uptake and hence actual concentration

of tryptophan in the brain, was being studied. Knott and Curzon (1972), demonstrating that fasted rats showed increased brain concentrations of tryptophan and 5HIAA, also reported that while the concentrations of free tryptophan and non-esterified fatty acids (NEFA) in the plasma rose, the total plasma concentration of tryptophan did not. Subsequently, Tagliamonte, Biggio, Vargiu and Gessa (1973) administered tryptophan loads to rats and showed that changes in the concentration of free serum tryptophan were more pronounced and longer lasting than changes in the total tryptophan concentration. They also reported that brain tryptophan and 5HIAA concentrations altered in proportion to changes in free, but not total serum tryptophan, rats fasted for twenty-four hours being found to have a lower concentration of total serum tryptophan but higher concentrations of free serum tryptophan and brain tryptophan and 5HIAA than animals which had been fed for two hours. It was therefore proposed that the brain concentrations of tryptophan and 5HT depended on the free, and not total concentration of serum tryptophan. In support of this, Perez-Cruet, Chase and Murphy (1974) reported that in humans, changes in the ratio of the concentrations of plasma free tryptophan to total plasma LNAA were highly correlated with changes in cerebrospinal fluid concentrations of tryptophan and 5HIAA, while the ratio of total plasma tryptophan:LNAA was not.

An initial investigation by Lipsett, Madras, Wurtman and Munro (1973) showed that administration of a glucose load resulted in a fall in the serum concentration of free tryptophan, although the concentration of albumin-bound tryptophan was unchanged. The fall in free tryptophan coincided with a drop in the plasma concentration of NEFA, adding oleic acid to the serum raising the proportion of unbound tryptophan to that of fasting subjects. It was postulated that the fall

in NEFA increased the availability of albumin, which then bound to tryptophan. This was in accord with the findings of Knott and Curzon (1972).

Further studies however, questioned the importance of the concentration of free, rather than bound tryptophan in the plasma or serum as a determinant of brain tryptophan concentrations. Madras, Cohen, Fernstrom, Larin, Munro and Wurtman (1973) also found a fall in the concentrations of free tryptophan and NEFA in the serum of the rat and a rise in the concentration of albumin-bound tryptophan after a glucose feed. In contrast to the results of Lipsett *et al.* (1973) however, an increase in total serum tryptophan and brain tryptophan concentrations was obtained, insulin injection having a similar effect. Rats fed a carbohydrate diet showed very similar results, supporting the proposed action of carbohydrate and insulin in increasing the binding of albumin to tryptophan by reducing its saturation with NEFA. However animals on a fat-containing diet showed a smaller fall in the serum concentrations of NEFA and free serum tryptophan, yet the same brain concentration of tryptophan. Thus it appeared that the concentration of free serum tryptophan could not predict changes in brain tryptophan as caused by feeding. Later work provided similar results and indicated that while the serum concentration of NEFA was a major regulator of the extent of binding of tryptophan to albumin, the concentration of free serum tryptophan did not necessarily determine brain tryptophan concentrations (Madras, Cohen, Messing, Munro and Wurtman, 1974). Fernstrom *et al.* (1976), reached essentially the same conclusions, finding a greater correlation of the brain concentration of tryptophan with the total serum tryptophan concentration or the ratio of this to

the sum of the plasma LNAA concentrations, than with the free serum tryptophan concentration or its ratio to the LNAA.

A role for free plasma tryptophan in determining brain concentrations of this amino acid was again suggested by Fernando, Knott and Curzon (1976), who found that chemical destruction of the insulin-secreting cells of the rat with streptozotocin increased the concentration of plasma free tryptophan but not the concentration of brain tryptophan itself. They did not however attribute the lack of alteration in the brain concentration of tryptophan to the observed lack of change in the total plasma tryptophan concentration. It was instead proposed that as reductions in the brain:plasma ratios of tyrosine and phenylalanine had been accompanied by a fall in the brain concentrations of these amino acids, the availability of tryptophan to the brain must have been increased for its brain concentration to have been maintained. This proposed increase in tryptophan availability was attributed to the increase in free plasma tryptophan resulting from raised plasma NEFA concentrations.

The argument for the influence of the plasma concentration of free tryptophan on the brain concentration of this amino acid was also supported by Hutson, Knott and Curzon (1976), who fed a high-fat diet to rats and showed that while the plasma concentrations of bound and total tryptophan fell, that of free tryptophan was unchanged, as was the brain tryptophan concentration. These workers suggested also that the reports of Madras and co-workers on the effect of feeding high-fat diets to rats had been a result of their using serum and not plasma, the NEFA concentrations being increased because of *in vitro* lipolysis during preparation of the serum and dialysis to separate free tryptophan. A greater amount of free tryptophan would hence have been

measured than might have been present in plasma, and erroneous data thus obtained.

The discussion as to whether the concentration of free or total plasma tryptophan was most effective in predicting the brain concentration of this amino acid was resolved when Yuwiler, Olendorf, Geller and Braun (1977) considered the amount of tryptophan stripped from albumin during a single capillary passage into the brain and concluded that both free and bound forms contributed to uptake, although uptake was greater in albumin-free solutions. Pardridge (1979) indicated that the binding capacity of the blood-brain barrier aided its competition with albumin for tryptophan, and thus more tryptophan was available for transport into the brain than might have been expected. In humans, the amount of tryptophan available for transport across the blood-brain barrier approximated the total plasma tryptophan concentration, not merely that which was not bound to albumin (Pardridge, 1983).

Determinations made in the rat (Leathwood, 1986) and man (Perez-Cruet, Chase and Murphy, 1974) indicate that allowing for the effects of both albumin and the competing LNAA might somewhat improve the prediction of the brain concentration of tryptophan or the cerebrospinal fluid concentration of 5HIAA. It is nevertheless apparent that the plasma concentrations of amino acids competing with tryptophan for uptake into the brain are of greater importance in determining the brain concentration of tryptophan than the degree to which that amino acid is bound to albumin in the plasma

1.5 Dietary amino acids and brain neurotransmitter concentraions

The brain concentrations of tryptophan, tyrosine and phenylalanine thus can be affected by their plasma concentrations in

relation to the concentrations of the LNAA competing with them for brain uptake. As the availability of the amino acid precursor appeared to be a major determinant of the rate of synthesis of the catecholamines and 5HT in the brain, investigations were made to determine whether changes in the concentrations of amino acids in the plasma and hence brain as induced by the diet or other methods, could bring about changes in the brain concentrations of these neurotransmitters.

a. Tryptophan and synthesis of 5HT

Fernstrom and Wurtman (1971a, 1972a) showed that the consumption of a carbohydrate diet or injection of insulin caused increased concentrations of tryptophan in the brain and plasma of the previously fasting rat which were accompanied by an increase in the brain concentration of 5HT. They also found that brain 5HT was dependent on plasma tryptophan concentrations, intraperitoneal loads of less than one twentieth of the daily intake elevating the brain concentration of 5HT within one hour (Fernstrom and Wurtman, 1971b), and suggested that physiological changes in the plasma concentration of tryptophan did influence the concentration of brain 5HT. Further work went on to show a correlation between the plasma ratio of tryptophan:LNAA concentrations and both the brain concentration of tryptophan and the combined concentrations of 5HT and 5HIAA (Fernstrom and Wurtman, 1972b). A meal of carbohydrate and fat accelerated synthesis of 5HT and increased concentrations of 5HT and 5HIAA in certain brain regions in the rat (Colmenares, Wurtman and Fernstrom, 1975).

The above investigations had been conducted on rats which had been fasted for twenty four hours however, and the effectiveness of the plasma tryptophan:LNAA ratio in predicting brain neurotransmitter concentrations found under other dietary circumstances has recently been

questioned. Ashley, Leathwood and Moennoz (1984) reported that rats which had been deprived of food for eight hours showed an increased brain concentration of tryptophan, but no change in the concentration of 5HT when fed a carbohydrate meal. Feeding an identical meal to animals which had been deprived of food for only three hours had no effect on the brain concentrations of either tryptophan or 5HT. Consumption of diets containing protein however, did reduce the brain 5HIAA concentration compared with that of those fed carbohydrate alone, the plasma tryptophan:LNAA ratio being inversely correlated to the dietary protein content. Nevertheless, the brain concentration of 5HT after feeding diets of differing protein content was independent of this ratio. It was thus suggested that the plasma tryptophan:LNAA ratio might only be correlated with the brain concentration of 5HT when the ratio itself was at an extreme value such as would be obtained during the feeding of diets specifically lacking or supplemented with some of the amino acids concerned.

In addition to an increase in brain tryptophan causing an increase in the brain concentration of 5HT under certain circumstances, tryptophan-deficient diets were shown to reduce the serum or plasma concentration of tryptophan and brain concentrations of tryptophan, 5HT and 5HIAA, supplements of tryptophan restoring the concentrations of these compounds (Biggio, Fadda, Fanni, Tagliamonte and Gessa, 1974; Zambotti, Carruba, Vincentini and Mantegazza, 1975; Fernstrom and Hirsch, 1975; Gibbons, Barr and Leibowitz, 1979). Alterations in dietary composition were thus able to bring about either increases or reductions in, or have no effect on, the brain concentrations of 5HT in the rat.

b. Tyrosine and phenylalanine and catecholamine synthesis

As has already been discussed in relation to the regulation of tyrosine hydroxylase activity, an increase in the brain concentration of tyrosine as achieved by its intraperitoneal administration in the rat was reported to result in an increase in the rate of catecholamine synthesis (Wurtman *et al.*, 1974; Gibson and Wurtman, 1977). Administration of phenylalanine caused an increase in the brain concentration of tyrosine without an accompanying rise in synthesis of the catecholamines (Wurtman *et al.*, 1974). In addition, some effect of changes in the diet on brain concentrations of catecholamines have also been shown. Shweiger, Warnhoff and Pirke (1985) subjected rats to conditions of acute or semi-starvation, the latter being achieved by feeding diets which were low in either their protein or carbohydrate content. Rats which had been completely fasted showed a reduced plasma ratio of tyrosine to the sum of the concentrations of the other LNAA, and a gradual fall in the brain concentrations of both tyrosine and NE. Those which were semi-starved maintained brain NE concentrations, but showed a reduction in the turnover of this neurotransmitter.

c. Effects of the diet on brain concentrations of both catecholamines and 5HT

Alterations in the brain concentrations of the catecholamines and 5HT were also reported as a result of other dietary manipulations. Green, Greenberg, Erickson, Sawyer and Ellison (1962) found that the dietary incorporation of excess phenylalanine depressed the rat brain concentration of 5HT and increased that of DA, the brain NE content being unaffected. Culley, Saunders, Mertz and Jolly (1963) and Boggs, Rosenberg and Waisman (1963) each confirmed the effect of excess phenylalanine (50-70g/kg) on brain 5HT concentrations, while

recent work with young rats showed reductions in 5HT and 5HIAA but no change in DA content during experimentally-induced hyperphenylalaninaemia (Taylor, Hommes and Stewart, 1983). The latter workers indicated that brain concentrations of tryptophan were unchanged by feeding excess phenylalanine and thus suggested that depletions in 5HT were likely to be caused by the competitive inhibition of tryptophan hydroxylase by phenylalanine rather than by a lack of substrate. In the chick also, excess dietary phenylalanine was shown to cause reductions in brain 5HT concentrations in birds of a broiler strain. The effect however was only seen to a lesser extent and only at a lower concentration of phenylalanine in two other strains tested. (Pscheidt and Tamimie, 1966).

A reduction in the brain concentrations of 5HT and DA in the rat was also reported as a result of feeding excess dietary leucine (Yuwiler and Geller, 1965; Geller and Yuwiler, 1967). Krishnaswamy and Raghuram (1972) indicated that the reductions in food intake and the brain concentration of 5HT caused by excess dietary leucine were each counteracted by a supplement of isoleucine. They suggested that isoleucine inhibited the leucine-caused rise in the degradative tryptophan pyrrolase (Rao and Gafforunnisa, 1972), so allowing increased synthesis of 5HT.

1.6. Amino acid imbalances, toxicities and antagonisms: causes and effects

It thus appears that alterations in the amino acid content of the diet and hence of the plasma and brain are able to influence neurotransmitter synthesis. More well-established effects of changes in the dietary amino acid pattern are alterations in growth and food intake, particularly those depressions in growth and food intake

observed to accompany the feeding of a diet which is distorted in its amino acid pattern. Several possible dietary amino acid patterns are illustrated in Fig. 3. Experimentation has demonstrated three main effects which may be obtained on alteration of the amino acid balance of a diet. These are termed amino acid imbalances, toxicities and antagonisms.

a. Amino acid imbalance

Harper (1958) defined amino acid imbalance as a case "in which the addition of a relatively small amount ....of an indispensable amino acid, or a mixture of such amino acids, or an unbalanced protein to a diet that is low in one or more amino acids causes a retardation of growth or some other adverse effect that can be prevented by the concomitant addition of small quantities of the limiting amino acids to the diet." Thus a relative deficiency of one or more essential amino acids is created by raising the dietary levels of others (Fig. 3.).

Such imbalances were shown in early work by Jackson, Sommer and Rose (1928) in which gelatin incorporated at a level of 150-350g/kg was confirmed as being an unsuitable source of nitrogen for rats, even if supplemented with the amino acids known to be absent or at relatively low concentrations. Hier, Graham and Klein (1944) attributed the poor growth rate of rats fed gelatin-containing diets to amino acid imbalance, the protein being deficient in methionine and tryptophan and having a high content of glycine and proline. Many more imbalances have since been demonstrated, including those with respect to lysine (Tews, Bradford and Harper, 1981), tryptophan (Sauberlich and Salmon, 1955; Savage and Harper, 1964) histidine (Sanahuja and Harper, 1962, 1963a) methionine and others in rats (Sauberlich, 1956; Harper,1959; Peng, 1979); and lysine, (Fisher, Griminger, Leveille and

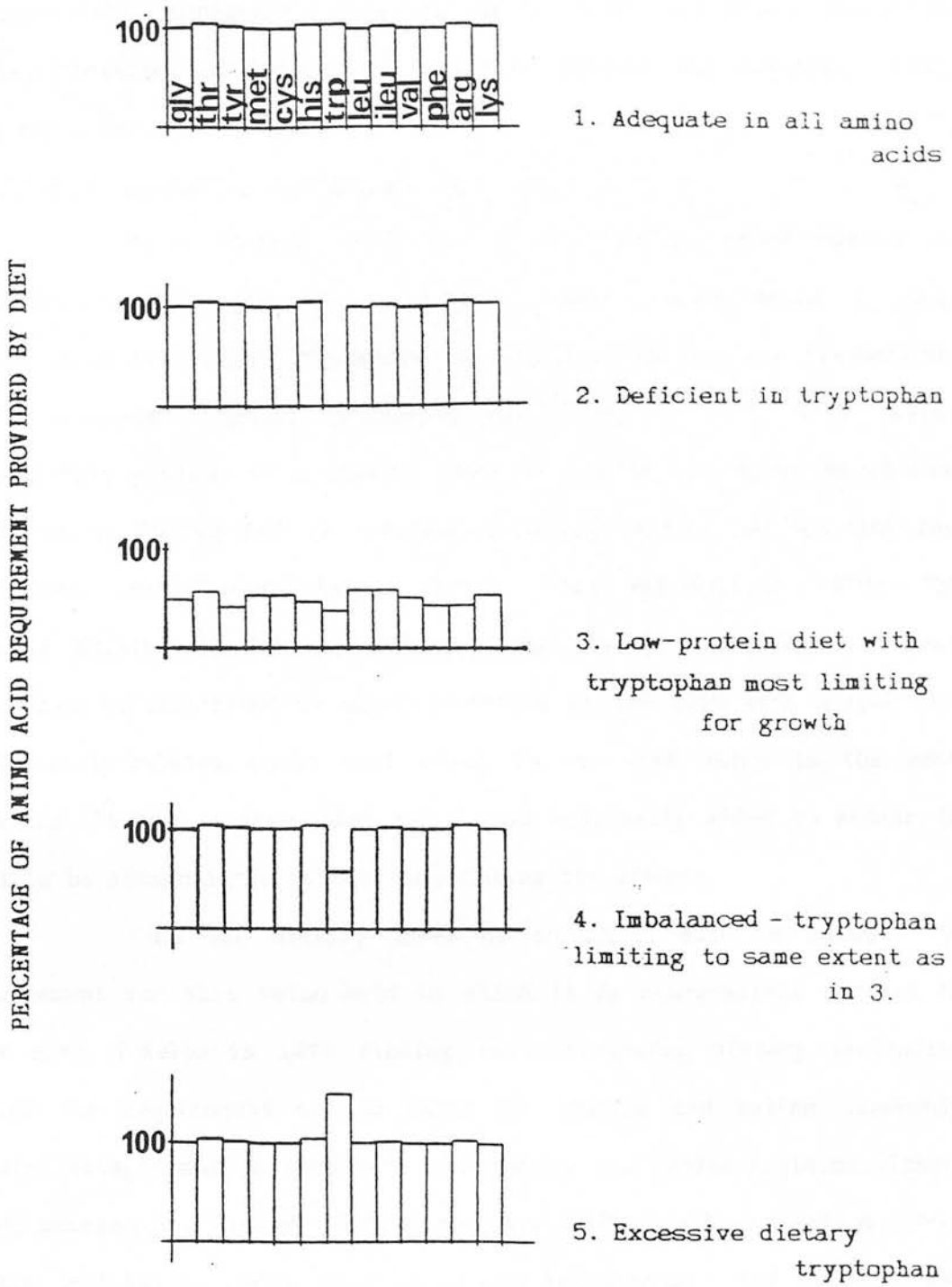


Fig. 3. Illustration of dietary amino acid patterns

Adapted from Harper, Benevenga and Wolhueter (1970)

Shapiro, 1960), threonine (Davis and Austic, 1982a) and others (Anderson, Combs, Groschke and Briggs, 1951; Fisher, Shapiro and Griminger, 1960; Hill and Olsen, 1963) in chicks.

b. Amino acid toxicity and antagonism

When certain amino acids are fed in great excess of requirements (Fig. 3.) their effect is toxic, there being a large variation in the degree of toxicity of different amino acids (Sauberlich, 1961; Muramatsu, Ogadiri, Morishita and Takeuchi, 1971). Cave (1978) showed that glycine at a dietary level of 30g/kg was toxic to chicks, while up to 20g/kg had no detrimental effect. Methionine toxicity has also been demonstrated (Hafez, Chavez, Vohra and Kratzer, 1978). The adverse effects of feeding excessive amounts of an amino acid may sometimes be alleviated by supplementation of the diet with a specific, structurally-related amino acid which is not that which is the most limiting. In such a case, that amino acid originally added to excess is said to be antagonistic to that alleviating its effects.

As the dietary level of an amino acid is raised, the requirement for that amino acid to which it is antagonistic appears to rise also, D'Mello in 1974 finding that increasing dietary isoleucine raised the requirement of the chick for leucine and valine. Commonly studied antagonisms in both rats and poultry are lysine-arginine (Jones, 1964; Boorman and Fisher, 1965; Anonymous, 1965; O'Dell and Savage, 1966; D'Mello and Lewis, 1970a; Kadirvel, Vohra and Kratzer, 1974; Wilburn and Fuller, 1975) and leucine-isoleucine-valine (Harper, Benton and Elvehjem, 1955; Benton, Harper, Spivey and Elvehjem, 1956; Rogers, Tannous and Harper, 1967; D'Mello and Lewis, 1970b). Possible cysteine-methionine (Featherstone, Rogler and Elkin, 1977) and phenylalanine-tryptophan (Elkin and Rogler, 1983) antagonisms have been suggested in poultry,

although the latter effect is reported between amino acids which are not structurally related and is perhaps therefore not an antagonism within the strictest sense of the original definition. Similarly in the rat, supplementation with threonine has been reported to alleviate the adverse effects of feeding excess dietary tyrosine (Alam, Boctor, Rogers and Harper, 1967).

c. Causes of reductions in growth

An effect common to amino acid imbalances, toxicities and antagonisms is a reduction in growth of the animal consuming a diet in which such a distortion of amino acid balance has occured, in many cases a decreased efficiency of food utilisation being reported (Muramatsu, Ogadiri, Morishita and Takeuchi, 1971; Sekiz, Scott and Nesheim, 1975; Smith and Austic, 1978; Elkin and Rogler, 1983; Davis and Austic, 1982b). Several proposals have been put forward to account for the observed growth-depressing effects.

i. Growth reductions caused by amino acid imbalance

The adverse effect on growth of feeding a diet which is imbalanced with respect to an amino acid appears in part to be due to the accompanying reduction in food intake. An early report indicated that the mean weight gain of rats fed a lysine-imbalanced diet was essentially similar to that obtained for those fed control diets after equalisation of intake by pairfeeding or forcefeeding (Hill and Olsen, 1965). Forcefeeding other imbalanced diets to rats stimulated growth also, without causing obvious abnormalities (Leung, Rogers and Harper, 1968a; Davis and Austic, 1982b). In addition, rats trained to feed in one daily meal decreased neither food intake nor growth unless the diet presented was much more severely imbalanced than one which would have had an effect on rats feeding *ad. lib.* (Leung, Rogers and Harper, 1968a).

Such work would support suggestions that the fall in food intake was primarily responsible for the reduced growth of animals fed a diet which was imbalanced in its amino acid content. However, Wethli, Morris and Shresta (1975) reported that a diet incorporating groundnut meal such that the minimal amino acid requirements were supplied by a high concentration of this imbalanced protein, did not support maximal lieweight gain or efficiency of food utilisation when fed to chicks. Some additional cause of the reduced growth caused by an amino acid imbalance thus existed.

The growth depression caused by a tryptophan imbalance has been suggested to be due to a wasting of tryptophan, the amino acid limiting for growth, via catabolism and excretion (Sauberlich and Salmon, 1955; Florentino and Pearson, 1962). Kumta and Harper (1961) reported that certain imbalances did cause increased urea production, but no increased catabolism or excretion of the limiting amino acid was observed during studies of imbalances with respect to threonine (Davis and Austic 1982b), or tryptophan (Wilson, Wortham, Benton and Henderson, 1962). Fisher, Griminger, Leveille and Shapiro, (1960) also found no evidence that lysine-imbalanced diets fed to chicks resulted in decreased efficiency of lysine utilisation, while others indicated that in a histidine-imbalanced diet, histidine itself was utilised with higher efficiency than in a balanced diet (Benevenga, Harper and Rogers, 1968; Soliman and King, 1969).

Cieslak and Benevenga (1984a,b,c) reported that amino acid imbalances with respect to lysine or threonine did not reduce the efficiency of nitrogen retention, and stated that the greatest part of the reduced growth of chicks fed diets which were imbalanced in this way was indeed due to their reduced food intake. In general, it appears

that the fall in food intake of animals fed a diet which is imbalanced with respect to an amino acid is the main cause of the accompanying depression in growth. The cause of the reduction in intake itself was indicated by Kumta and Harper (1961) not to be due to slowed gastric emptying.

ii. Growth reductions caused by amino acid toxicity and antagonism

It has been reported that the reduction in growth seen as a result of a dietary excess of an amino acid -whether this is an effect of toxicity or antagonism- is not merely due to the accompanying reduction in food intake. Forcefeeding the rat with a diet containing excess tyrosine has been shown to give a transitory weight gain only (Boctor and Harper, 1968). In addition, D'Mello and Lewis (1971) reported that even when chicks receiving a control i.e. balanced diet were pairfed with those consuming diets containing excess lysine or leucine, there was still an appreciable difference in weight gain between the latter groups of chicks and those fed the control diet. Forcefeeding chicks with diets containing excessive amounts of leucine or lysine also did not produce growth comparable to that of those fed a control diet (Austic and Scott, 1975; Calvert, Klasing and Austic, 1982). A report that the reduction in weight gain caused by excessive dietary lysine reached significance before the depression in food intake would support there being some additional factor contributing to the observed growth depression (D'Mello and Lewis, 1971).

Further causes of the depressed growth resulting from the consumption of a diet containing an excess of one amino acid have been investigated. Alam *et al*, (1967) reported that the alleviation of the effects of excess tyrosine by threonine was due to an increase in tyrosine catabolism, while Datta and Ghosh (1977) indicated that a

tyrosine-caused increase in the catabolic threonine dehydratase induced a relative deficiency of threonine which was alleviated by a supplement of this amino acid. For rats fed excess methionine, glycine addition was shown to partially alleviate the growth depression caused and also to increase urinary methionine , while the addition of methionine to a diet increased urinary glycine (Adkins, Boffman and Wertz, 1968). Thus it appears that an amino acid in dietary excess possibly may produce its adverse effects by increasing the elimination of certain other amino acids via catabolism and excretion.

Recent work has suggested that the growth-depressing effect of excess leucine is to a large extent caused by the reduced intake of the diet, but is also due to an increase in catabolism of the branched-chain amino acids which limits the availability of isoleucine and valine (Calvert, Klasing and Austic, 1982). An increase in the oxidation of isoleucine in rats (Phansalkar, Norton, Holt and Snyderman, 1970), and both this amino acid and valine in chicks (Smith and Austic, 1978; Calvert, Klasing and Austic, 1982) was reported to be caused by excessive dietary leucine, probably partially due to reported increases in the activity of the degradative BCAA aminotransferase in the muscle of the chick (Smith and Austic, 1978) and hepatic branched-chain α-keto-acid dehydrogenase in rats (Wolheuter and Harper, 1970). No increase in the excretion of isoleucine and valine was apparent on feeding excess dietary leucine however (Clark, Yamada and Svensend, 1968).

After Cittadini, Pietropaulo, De Cristofaro and D'Ayjello-Caraciollo (1964) reported that intraperitoneal administration of a large dose of lysine to rats caused an inhibition of liver arginase activity, Jones, Wolters and Burnett (1966) suggested that high levels of dietary

lysine interfered with utilisation of arginine in the urea cycle and thus increased the requirement for this amino acid. However, that in rats the antagonism of arginine by lysine was due to the involvement of arginine in the production of urea cycle intermediates was contradicted by Ashley and Anderson (1977a). Excessive dietary lysine was reported to increase kidney arginase activity and urea excretion (Jones, Petersburg and Burnett, 1967, Austic and Nesheim, 70), a great excess of lysine increasing urinary arginine (Nesheim, 1968; Boorman, 1971) but a moderate one having no such effect (Austic and Scott, 1975).

The fall in food intake appears to be responsible for a greater proportion of the reduction in growth accompanying an amino acid imbalance than that accompanying the consumption of diets containing an excess of one amino acid. The observed reduction in food intake caused by a dietary excess of an amino acid remains however, a cause of at least some portion of the growth depression observed. No definite cause of the reduction in food intake as caused by feeding a diet containing an excess of an amino acid is clear, Spolter and Harper (1961) reporting that the reduced food intake caused by excess leucine was not due to a change in nitrogen absorption, retention, or the rate of gastric emptying.

The subjects of dietary amino acid imbalances, toxicities and antagonisms, their effects and possible causes have been widely reviewed (Harper, 1956; Salmon, 1958; Harper, Benevenga and Wolhueter, 1970; Benevenga and Steele, 1984).

1.7. Dietary choice

Consumption of a diet may therefore be reduced if its amino acid composition is distorted in one of the various ways described previously. Rats will preferentially select a balanced diet, but if none

is available, will choose a protein-free diet rather than one which is imbalanced or contains an excess of an amino acid (Sanahuja and Harper, 1962; Rogers, Tannous and Harper, 1967). The adverse effect of an amino acid added to the diet at a high concentration may however be reduced if the dietary protein concentration or the relative concentrations of all other indispensable amino amino acids is raised (Harper, Becker and Stucki, 1966; Muramatsu, Ogadiri, Morishita and Takeuchi, 1971).

While the ability of animals to alter their intake of a diet which was grossly distorted in its amino acid pattern had been shown in early investigations, more recent work suggested that a specific mechanism might operate for the control of protein intake within a definite range. Musten, Peace and Anderson (1974) allowed weanling rats to select from a protein-free diet and one containing either casein or gluten. They reported that the intake of protein was constant in relation to the total amount of energy consumed, and that this was increased when the lower-quality gluten was fed rather than casein. Gluten-fed rats maintained their protein intake even when the gluten-containing diet was diluted, and exposure of the animals to cold increased their intake of the protein-free diet only. It was proposed therefore that the rat was able to independently regulate its protein and energy intakes.

Some support for the ability of the animal to regulate its protein intake was supplied by Ashley and Anderson (1975a) who showed that the improvement of the nutritional quality of a gluten-containing diet by addition of lysine, the amino acid which was most limiting for growth, resulted in a reduction in the quantity of this diet selected and an increase in both energy intake and growth. However, further attempts to improve the amino acid balance of this diet were without effect

(Ashley and Anderson, 1975a, 1977a). Later work by Peters, Nemetz, Tews and Harper (1983) found no strict control of protein intake, this not being maintained at a constant proportion of the total energy consumed but lying within a wide range. Both weanling and adult groups of rats were allowed to select between diets of differing protein content by Leathwood and Ashley (1983), who reported that while most animals tended to consume a constant amount of protein, the variation between individuals was large. In addition, when dietary protein contents were altered, a shift in the amount of protein consumed by weanling rats occurred. It thus appeared that the quantity of protein consumed was perhaps not regulated strictly, according to the needs of the animal and the nutritional quality of the protein available.

The effect of dietary protein content on the food intake of an animal is therefore not entirely clear. Dilution of the protein content of a diet has been shown to increase its intake in both rats (Musten, Peace and Anderson, 1974) and poultry (Savory, 1984; Cherry, Young and Jones, 1984), while intake of a high-protein diet is initially depressed (Peng, Meliza, Vavich and Kemmerer, 1974) and rises as the capacity of the animal to degrade amino acids increases (Peters *et. al.*, 1983). The only other situations where a definite, well-regulated response to dietary protein is apparent appear to be the depressions in intake seen as a result of consumption of diets which are imbalanced in their amino acid content or incorporate an amount of one amino acid sufficient to cause an antagonistic or toxic effect. It may be therefore that the intake of protein affects the feeding mechanisms only in conditions of nutritional stress. It is perhaps necessary at such a point to alter intake of the diet in order to reduce adverse effects likely to occur as a result of its maintenance at the original level.

1.8. Plasma amino acid concentrations and food intake

The role of plasma amino acids in the effects of imbalances and toxicities has been investigated, as a change in amino acid concentrations in the plasma could provide some sort of trigger for a mechanism by which food intake might be altered. The plasma concentration of the most limiting amino acid has been shown to fall soon after ingestion of an imbalanced diet by rats or chicks (Kumta and Harper, 1962; Sanahuja and Harper, 1963b; Sanahuja, Rio and Lede, 1965; Zimmerman and Scott, 1965; Leung, Rogers and Harper, 1968b; Tews and Harper, 1982), while excess leucine has been shown to deplete plasma isoleucine and valine in rats (Tannous, Rogers and Harper, 1966; Clark, Yamada and Swendsend, 1968) and chicks (D'Mello and Lewis, 1970b). Peng and Harper (1969) found that the depression in food intake which was caused by a histidine imbalance was proportional to the total plasma concentrations of indispensable amino acids and also the degree of abnormality of the plasma amino acid pattern. Force-feeding an imbalanced or high-protein diet resulted in increased plasma total amino acid concentrations and a distorted amino acid pattern in both the plasma and brain of rats (Peng, Tews and Harper, 1972). Ingestion of the preferred protein-free diet appeared to restore plasma amino acid concentrations more nearly to those of rats fed a control diet (Leung, Rogers and Harper, 1968b; Peng, Meliza, Vavich and Kemmerer, 1975). Harper, Leung, Yoshida and Rogers (1964) suggested that the fall in the level of the most limiting amino acid as caused by an imbalance resulted in the blood amino acid pattern resembling that of an animal fed a diet which was much more deficient in that amino acid, falsely indicated to an appetite-regulating mechanism that the diet was actually

more deficient in that amino acid than was the case and so caused a reduction in food intake.

It thus appeared that food intake regulatory mechanisms might indeed be sensitive to alterations in the circulating levels of plasma amino acids, a reciprocal relationship between serum amino acid levels and food intake having been proposed by Mellinkoff, Frankland, Boyle and Greipel (1955). Anderson, Benevenga and Harper (1968) showed a correlation between the adaptation of rats to a high-protein diet, a fall in their plasma amino acid levels and the restoration of food intake. However, while ingestion of a diet could bring about a change in the plasma concentrations of amino acids directly, the identity of a mechanism by which such a change might result in an alteration in food intake remained unclear.

1.9. Involvement of the brain in the regulation of appetite

The hypothalamus was first implicated as being involved in appetite regulation when tumours at the base of the human brain were found to cause hyperphagia and obesity (Froelich, 1901). Lesions of the ventromedial hypothalamus (VMH) of the rat were shown to have a similar effect (Hetherington and Ranson, 1940; Anand and Brobeck, 1951), Morgane (1961) indicating that electrical stimulation of certain lateral hypothalamic regions could provoke various eating reactions in the satiated rat. The involvement of lateral hypothalamic regions in the regulation of food intake was supported by Bernardis, Luboshitsky, Bellinger and McEwen (1982). Studies in the chick reported that hypothalamic lesions caused either aphagia or hyperphagia and obesity, depending on their location (Lepkovsky and Yasuda, 1966; Smith, 1969a). The structure of the brain of the chick is illustrated in Figs. 4. and 5.

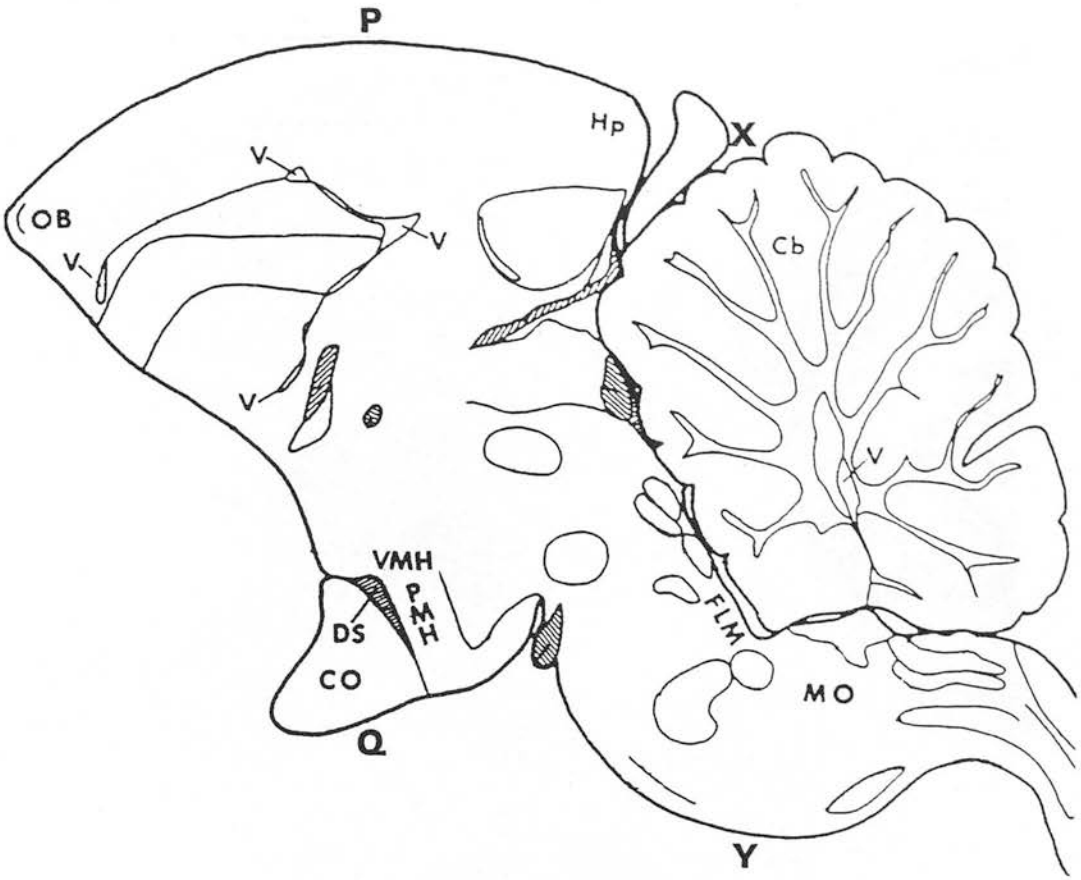


Fig 3. Diagram of the brain of the chick

(from Youngren and Phillips, 1978)

Cb	Cerebellum
Hp	Hippocampus
MO	Medulla oblongata
OB	Olfactory bulb
PMH	Posterior medial hypothalamus
V	Ventricle
VMH	Ventromedial hypothalamus

Other abbreviations according to Youngren and Phillips (1978)

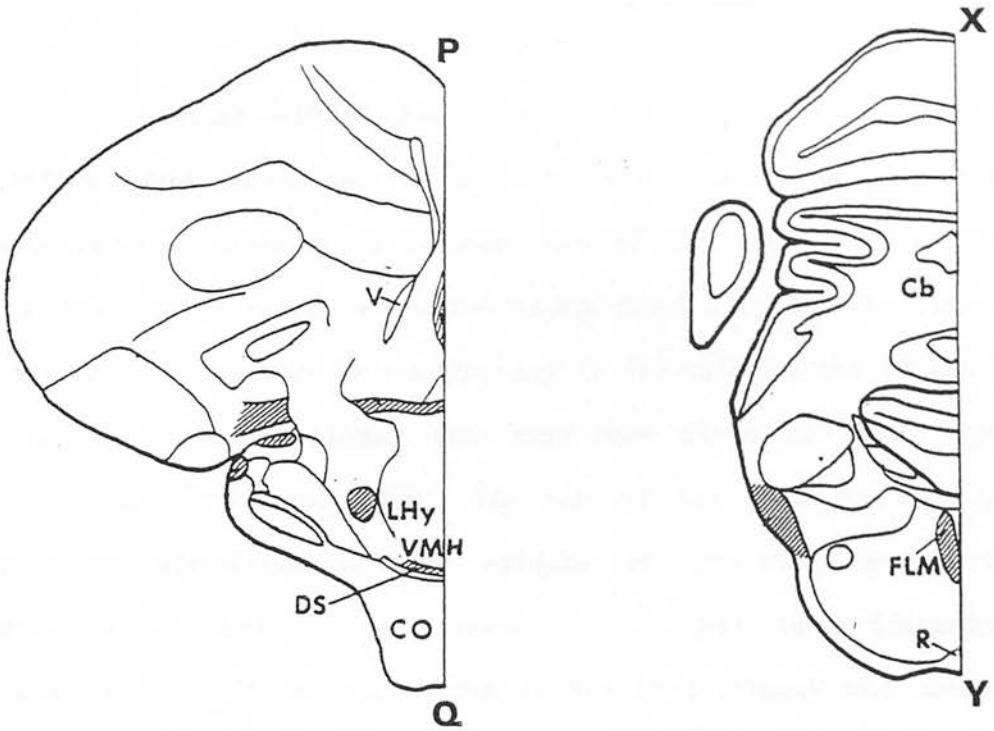


Fig. 4. Longitudinal sections through chick brain along planes P-Q and X-Y indicated in Fig. 3. (Half sections shown)

Cb Cerebellum

LHy Lateral hypothalamus

R Raphe Nucleus

V Ventricle

VMH Ventromedial hypothalamus

Other abbreviations according to Youngren and Phillips (1978)

Stellar (1954) had proposed that an excitatory and an inhibitory hypothalamic centre regulated food intake, assuming that the effects observed when brain lesions were produced were in fact due to the destruction of a regulatory mechanism which responded to changes in extracellular amino acid levels. However in the rat, lesions of the brain external to the hypothalamus have also been shown to affect appetite (Grossman and Grossman, 1963) The role of the brain in food intake control as elucidated by the effects of lesioning or electrical stimulation of various brain areas has been discussed by Grossman (1968). The specific role of the hypothalamus was considered by Rabin (1972), and the existence of a dual hypothalamic centre for the regulation of food intake challenged.

The effect of brain lesions on the ability of an animal to respond to a diet which is imbalanced with respect to an amino acid or contains a high concentration of one amino acid has been studied widely. Nasset, Ridley and Schenk (1967), reported that VMH-lesioned rats lost the ability to discriminate between diets which were balanced or imbalanced with respect to their amino acid content, intake and weight gain being similar in each case. However, Sharrer, Baile and Mayer (1970) studied the effects of toxic, imbalanced and high-protein diets on hypothalamic-hyperphagic, normal and recovered aphagic rats and indicated that hyperphagic rats showed a depression of food intake below that seen in normal and recovered individuals. They suggested that the results obtained by Nasset *et al.* (1967) were due to their employing animals which were still in the dynamic phase of hyperphagia, eating more and weighing less than those at the later static phase which had been studied by Scharrer *et al.* (1970).

Further work by Arakawa, Standal and Beaton (1971) found that lesioned hypothalamic-hyperphagic rats preferred, but intact rats showed no preference for, a protein-free diet when offered a choice between that and a diet into which had been incorporated an imbalanced mixture of amino acids at a concentration of 20g/kg. It had previously been shown that intact rats preferred a protein-free diet when allowed to choose between that and a diet containing an imbalanced amino acid mixture at a concentration of 38g/kg (Sanahuja and Harper, 1962, 1963a). This implied that lesioned rats were somewhat more sensitive to the milder imbalance introduced in this experiment. Preference for a diet which was balanced in its amino acid content over one which was imbalanced was apparent in both lesioned and intact animals. As the plasma amino acid patterns were similar in both lesioned and control groups, Arakawa *et al.* (1971) proposed that the VMH did not therefore constitute a regulatory centre for control of intake of diets which were imbalanced with respect to an amino acid.

Lesions of brain areas other than the hypothalamus were also shown to affect the response of the rat to diets which were imbalanced with respect to an amino acid. Lesions of the rat prepyriform cortex (an area very close to the olfactory bulb) which did not cause increased consumption of a diet which was balanced in its amino acid content, were found to cause a loss of discrimination between imbalanced and control diets and a preference for an imbalanced rather than a protein-free diet. However, a depression in intake still occurred when a high-protein diet was fed (Leung and Rogers, 1971). Rats with hippocampal lesions consumed a diet having a high casein content to an extent comparable to that of intact animals and still reduced their intake of diets which were imbalanced or deficient with respect to

threonine, but a greater degree of adaptation to the imbalanced diet was indicated (Leung and Rogers, 1979). Adaptation to a high-protein diet was again similar to that of normal rats.

Various lesions in different areas of the brain have thus been found to affect the ability of the animal to regulate its intake of diets which are balanced or distorted in their amino acid content. It appears that the effects of such lesions are more likely to be due to the interruption of particular networks of neurones than the destruction by each lesion of a specific, localised brain centre which regulates one aspect of feeding behaviour (Stricker and Zigmond, 1984). Nevertheless, evidence to support the involvement of the brain in the regulation of food intake in response to alterations in plasma amino acid concentrations is strong. Together with the above investigations of the effects of brain lesions, the injection of balanced mixtures of amino acids into the hypothalamus was shown to inhibit feeding in rats (Panksepp and Booth, 1971). In addition, infusion of the relatively deficient amino acid into the carotid artery of rats and cockerels fed an imbalanced diet was reported to alleviate its adverse effects (Leung and Rogers, 1969; Tobin and Boorman, 1979), while infusion of lysine into the carotid artery of cockerels fed a diet which was balanced in its amino acid content caused a much more marked fall in food intake than its infusion into the jugular vein (Tobin and Boorman, 1979). These data all imply that the brain is involved in the regulation of food intake via a mechanism which is sensitive to plasma amino acid concentrations.

1.10. Brain neurotransmitter concentrations and food intake

a. Catecholamine concentrations and food intake

Many reports exist which suggest that certain of the catecholamines of the brain are able to affect food intake. Early work by Grossman (1962a,b) showed that vigorous and prolonged eating in food-satiated animals was caused by the administration of E or NE into the hypothalami of rats. This eating response was reported to be potentiated by the drug desmethylimipramine which blocked the neuronal reuptake of NE (Booth 1968), although others reported that this drug exerted no effect in rats unless they had previously been fasted (Montgomery, Singer and Purcell, 1969). A supression of feeding by the injection of NE into the mid-brain region was reported by Margules (1969), Kruk (1973) reporting that injection of NE into the rat brain caused increased food intake while DA injection caused a decrease. A specific effect of NE on meal size was suggested by Ritter and Epstein (1975), who showed that its infusion into the rat hypothalamus at less than 1% of endogenous brain concentrations increased meal size when administered at the beginning of eating, but had no effect if given between meals. In poultry, intraventricular injections of E and NE were shown to increase food intake in the chick (Denbow, Cherry, Siegel and Van Krey, 1981) but reduce it in the turkey (Denbow, 1983), DA having no effect.

It thus appeared that some sort of relationship between brain concentrations of NE and possibly the other catecholamines and food intake might exist. Investigations in the rat demonstrated that destruction of brain dopaminergic fibres by injection of 6-hydroxydopamine prevented the hyperphagia caused by VMH lesions, and

that these fibres appeared to be essential for the control of food intake (Rowland, Marshall, Antelman and Edwards, 1979).

The administration of various anorectic drugs also provided evidence that certain brain neurotransmitters might be influential in the regulation of food intake, Blundell and Leshem (1973) finding that the anorectic drugs amphetamine and fenfluramine had an effect only when injected into the lateral, and not the ventromedial region of the hypothalamus. The action of the anorectic drug amphetamine was suggested to be mediated by the catecholamines of the brain, Ahlskog and Hoebel (1973) showing that treatment with 6-hydroxydopamine and electrical destruction of noradrenergic terminals supplying the hypothalamus caused hyperphagia and suppressed the effect of amphetamine. The lack of effect previously reported by Samanin, Ghezzi, Valzelli and Garattini (1972), using 6-hydroxydopamine to reduce brain NE and DA, was attributed to the depletions in these catecholamines being insufficiently great.

Other evidence supporting the existence of a role for the catecholamines in the control of food intake was provided by Sauter, Goldstein, Engel and Ueta, 1983). Systemic or intraventricular administration of insulin, a hormone known to increase food intake, was shown to increase the turnover but not actual concentrations of E and NE in the hypothalamus and medulla oblongata of the rat. A dose-related release of E, NE, and DA from hypothalamic slices was obtained during incubation with insulin, indicating that its action might be via these compounds. In addition, increased concentrations of NE were reported in certain hypothalamic regions of rats trained to eat within a restricted time, or taken at their highest feeding rate (Van der Gugten, De Kloet, Versteeg and Slangen, 1977). Genetically obese mice showed greater

concentrations of NE and DA in the hypothalamus and forebrain hemispheres (Lorden, Oltmans and Margules, 1975), while genetically obese rats were also reported to have both higher and lower concentrations of these compounds in various areas of the brain (Cruce, Thoa and Jacobowitz, 1976).

Thus NE and possibly the other catecholamines are implicated as being involved in the control of food intake. Pharmacological evidence that each of these compounds may have a role in both the stimulation of the eating process and its supression has been discussed by Hoebel (1977).

b. 5HT concentrations and food intake

Food intake was also shown to be reduced by the hypothalamic injection of 5HT into rats (Goldman, Lehr and Friedman, 1971). A similar effect was obtained on intraventricular administration of 5HT into the fully-fed chick, the effect being removed in birds which were fasted for twenty-four hours (Denbow, Van Krey and Cherry, 1983).

The study of drugs which caused an alteration in food intake also supported the existence of some mechanism involving 5HT which regulated food consumption. The anorectic action of an intraperitoneal dose of the drug fenfluramine was antagonised by mid-brain raphe lesions which were found to deplete forebrain concentrations of 5HT (Samanin *et al.*1972), while a drug blocking 5HT receptors in the brain showed strong antagonism to fenfluramine but was only weakly or not at all antagonistic to the action of amphetamine (Jespersen and Scheel-Kruger, 1973). Blundell and Leshem (1975) lent support to there being a role for 5HT in the anorectic effect of fenfluramine, the drug being reported to increase serotinergic transmission (Fuller, Snoddy and Hemrick, 1978; Trulson and Jacobs, 1976).

D,L-parachlorophenylalanine (pCPA) is an inhibitor of 5HT synthesis, depleting the brain of 5HT (Koe and Weissman, 1966). Intraventricular injection of this drug was shown to cause hyperphagia (Breisch and Hoebel, 1975), although it was also found that oral or intraperitoneal administration caused a reduction in protein intake (Ashley and Anderson, 1977b; Ashley, Coscina and Anderson, 1979). Hoebel, Zemlan, Trulson, Mackenzie, Ducret and Norelli (1978) showed that the hyperphagia caused by intraventricular pCPA was reversed by intraperitoneal administration of 5-hydroxytryptophan (5HTP), the immediate precursor of 5HT, which restored brain concentrations of this neurotransmitter. However, int raperitoneal administration of pCPA did not antagonise the action of fenfluramine, but enhanced it in rats on a six-hour feeding schedule (Hoebel *et al.*, 1978). The reason for this apparent conflict was unclear, except that hyperphagia caused by pCPA might in fact involve factors other than serotinergic systems. Nevertheless, a role for 5HT in the control of food intake would appear to be supported. The evidence for this has been considered by Hoebel (1977).

Data regarding the effects of injection of the catecholamines and 5HT into the brain, together with information obtained from the action of anorectic drugs, indicated that these neurotransmitter compounds might indeed exert some influence over the food intake of an animal. Blundell, Latham and Leshem (1976) suggested that the catecholamines and 5HT had different roles in the regulation of food intake, their effects being on hunger and satiety respectively. Plaznik, Danysz, Kostowski, Bidzinski and Hauptmann (1983) also suggested differing roles for these neurotransmitters, finding that injection of adrenergic agonists (compounds increasing the release of NE

or interacting with NE receptors), including NE itself, tended to deplete the forebrain of the 5HT metabolite 5-hydroxyindoleacetic acid (5HIAA), while the converse was true for some adrenergic antagonists. A possible mutual antagonism of the activities of 5HT and the catecholamines was suggested.

More recent consideration of the possible functions of the brain concentrations of the catecholamines has suggested that these compounds are involved in general aspects of behavioural arousal rather than the specific regulation of food intake. Depletion of the 5HT content of the rat brain appears to have effects which are not solely confined to the control of eating, an impairment of thermoregulation and also water intake having also been reported (Myers, 1975). Lesions of DA-containing neurones may impair all voluntary activities, a greater stimulus being required in order to produce a particular response and changes in food intake merely being a reflection of the overall alertness and responsiveness of the animal. This has been discussed by Stricker and Zigmond (1984). Further study of the factors affecting neurotransmitter concentrations of the brain seems to be required.

1.11. Dietary amino acid content, plasma amino acid ratios, brain neurotransmitter concentrations and food intake

It is therefore apparent that the amino acid content of a diet consumed is able to affect the plasma amino acid pattern. Under appropriate circumstances an alteration in this may bring about changes in the amino acid content of the brain, which in turn can result in changes in the concentrations or rates of turnover of one or more of the catecholamine neurotransmitters and 5HT. Some evidence also exists that these compounds themselves may be involved in the regulation of food intake. With these considerations in mind, investigations were made to

determine whether dietary-induced changes in plasma amino acid ratios and brain concentrations of the catecholamines and 5HT might be related to the food intake response of the animal.

Ashley and Anderson (1975b) reported a negative correlation between the protein intake of rats selecting food from two diets differing in protein content and the plasma ratio of tryptophan:LNAA, and also positively correlated the ratio of plasma tyrosine to phenylalanine with energy intake (Anderson and Ashley, 1977). They suggested that changes in these plasma ratios triggered mechanisms which regulated protein and energy intake respectively. Further work on rats by Fernstrom, Wurtman, Hammarston-Wiklund, Rand, Munro and Davidson (1979), found that the total plasma LNAA concentration was proportional to the protein content of the diet fed and that the ratio of plasma tryptophan, tyrosine or phenylalanine to total plasma LNAA fell as protein content rose. This was taken to imply that the ingestion of diets differing in their carbohydrate and protein content would produce different effects on the brain concentrations of the catecholamines and 5HT.

A relationship between the brain concentration of 5HT and the regulation of food intake was suggested by Ashley and Anderson (1977b), who showed that the protein intake of rats allowed to select protein and carbohydrate independently was reduced by administration of pCPA -this being known to deplete the brain of 5HT. In addition, Wurtman and Wurtman (1977, 1979) reported that the effect of fenfluramine in rats allowed to select protein and carbohydrate independently was to suppress their intake of carbohydrate but not protein. This anorectic drug stimulates the release of 5HT (Trulson and Jacobs, 1976). The two effects implied that under conditions which resulted in increases or

decreases in the brain concentration and presumably release of 5HT, the intake of carbohydrate and protein respectively were reduced. However when Weinberger, Knapp and Mandell (1978) injected fasted rats with tryptophan loads, a rise in brain concentrations of tryptophan, 5HT and 5HIAA was not accompanied by any change in intake of the single diet presented. Anderson (1979) suggested that unless distortions in plasma and brain amino acid patterns were gross, the regulation of energy intake took priority over control of protein intake, and thus the intake of a single diet would be unchanged by such a load. In support of this proposal, rats selecting protein and energy separately were reported to decrease their protein intake when the tryptophan content of the diet was increased (Woodger, Sirek and Anderson, 1979). Such an effect however, would tend to conflict with the reported fall in protein intake under conditions of 5HT depletion. This might be explained by there being a requirement for the brain concentration of 5HT to lie within a particular concentration range and food intake being depressed as a result of either a rise or fall in concentration beyond certain limits.

Arimanana, Ashley, Furniss and Leathwood (1984) indicated that administration of a glucose or tryptophan load to rats caused an increase in the brain tryptophan concentration, glucose also increasing the brain concentrations of 5HT and 5HIAA. A mixture of the LNAA reduced the concentrations of all three compounds in the brain. When subsequently allowed to select from a protein-free and a casein-containing diet, rats which had previously been given tryptophan or glucose selected a greater proportion of their energy intake as protein than those given the LNAA load. This was consistent with a prediction that an increase in 5HT synthesis should increase preference specifically for protein.

Other studies however, obtained results which conflicted with those just reported. Peters and Harper (1981) found no correlation between protein consumption and the plasma ratio of tryptophan:LNAA or brain concentrations of 5HT; or between energy intake and plasma ratios of tyrosine:phenylalanine, tyrosine:total LNAA or brain concentrations of NE and DA. A strong correlation between protein intake and the total plasma concentrations of the branched-chain amino acids was however reported. Li and Anderson (1982) studied rats allowed to select protein and carbohydrate separately and stated that while the plasma ratio of tryptophan:LNAA and brain concentrations of tryptophan and 5HT and 5HIAA were inversely correlated to the protein content of the meal previously selected, this appeared to be a consequence of the food consumed and not a determinant of subsequent protein intake.

Leathwood and Ashley (1983) suggested that while the proportion of the dietary energy selected as protein was inversely correlated with the plasma tryptophan:LNAA ratio, the level of protein or energy intake, or the brain concentrations of 5HT and 5HIAA were not. They stated that the tryptophan:LNAA ratio was only reliably influential in predicting brain 5HT when it was high, as induced by the feeding of diets which were totally lacking in protein or specifically altered in their content of tryptophan or the other LNAA. The brain concentrations of 5HT and 5HIAA did correlate well however with energy intake. Investigations by Peters and Harper (1985) showed that the brain concentrations of NE, DA or 5HT of rats receiving different dietary concentrations of protein were not correlated with the level of dietary protein, protein intake or total food intake, although brain concentrations of HVA and 5HIAA were inversely correlated with the protein intake of these animals and brain tryptophan and tyrosine

concentrations varied inversely with the plasma concentrations of the branched-chain amino acids.

Fernstrom, Fernstrom, Grubb and Volk (1985) supported the view that none of these measurements was correlated with the long-term intake of protein. Having fed rats diets of differing protein concentrations, they reported that while the serum tryptophan concentration was lowest in rats fed the diet having the lowest protein content, the serum tryptophan:LNAA ratios, brain tryptophan and 5-hydroxyindole concentrations of animals fed the different diets did not differ.

Thus while changes in the dietary amino acid content may under some circumstances influence the synthesis of certain neurotransmitters in the brain, evidence for a mechanism involving the catecholamines or 5HT in the tight regulation of the intake of food, whether as a single diet or as independently-selected protein and carbohydrate, is weak. There is little to suggest that the consumption of a protein-carbohydrate diet by non-fasting animals will greatly affect their brain concentration of 5HT, although less data are available on possible effects on brain catecholamines. Several reviews of the possible interrelationships between food intake, plasma amino acid levels and brain neurotransmitters have been published (Fernstrom, 1976, 1983; Hoebel, 1977; Booth, 1978; Barrett, 1978; Anderson, 1979, 1981; Peters and Harper, 1981; Anderson and Johnston, 1983).

1.12 Significance of changes in brain concentrations of the catecholamines and 5HT

If brain concentrations of the catecholamines and 5HT do in fact have a functional significance, a change in these concentrations should be reflected in an alteration of the activity of the

catecholaminergic or serotinergic neurones. Alonso, Agharanya and Wurtman (1980) reported that the administration of tyrosine to the rat caused an increase in urinary excretion of the catecholamines without depleting their concentrations in the tissues, this being consistent with their increased synthesis and release by catecholaminergic cells outside the central nervous system. However, no reports of such increases in release of the catecholamines from neurones of either the peripheral sympathetic nervous system or the central nervous system appear to be available.

Administration of tryptophan to the rat has been reported to slow the firing frequency of serotinergic neurones (Gallager and Aghajanian, 1976). Trulson (1985) reported however, that while feeding rats diets which varied in their proportions of tryptophan and LNAA produced significant differences in their brain concentrations of 5HT and 5HIAA, no change in activity of the 5HT-containing raphe cells was apparent. Injection of radiolabelled tryptophan indicated that an increase in the concentration of radioactive 5HIAA occurred without increased release of labelled 5HT into the ventricles of the brain. This implied that no alteration in the functioning of serotinergic neurones had occurred. Thus from the little data available, the actual functional significance of any changes in the brain concentrations of the catecholamines and 5HT is uncertain.

1.13 Background to current investigations

For effective overall control of all bodily processes, the metabolism of the brain must be easily altered by changes in the environment external to it. It is clear that the amino acid content of the diet has a role to play in the regulation of food intake, and that this regulation is mediated by some mechanism of the brain. Very little

is known about the relationship of the diet to the functioning of the central nervous system however. Under certain conditions, changes in the amino acid content of the diet may bring about alterations in the concentrations of the catecholamines and 5HT in the brain, but under normal feeding the effect appears to be less extreme. Greatest alterations in the brain content of these neurotransmitters are seen as a result of feeding diets which are specifically supplemented with, or lacking in, one or more of the amino acids which are either precursors of these compounds or compete with the precursor amino acids for brain uptake.

Both excessive dietary phenylalanine and leucine have been reported to reduce the brain concentration of 5HT (section 1.5c), but there is little else known about the effects of changes in dietary amino acid content such as cause the phenomena of amino acid imbalances, toxicities and antagonisms, on the brain concentrations of the neurotransmitters under discussion. These extreme distortions in the dietary amino acid pattern are perhaps more likely to affect the brain concentrations of the catecholamines and 5HT than the small changes in amino acid content and pattern which might be encountered when feeding experimental diets of different types which are generally well-balanced in their amino acid content. The possibility of one or more of the catecholamines and 5HT playing some role in the control of food intake also remains. Many previously-mentioned reports have been made of the adverse effects on growth and food intake and various metabolic effects of feeding diets which are distorted in their amino acid balance, but the mechanism by which this distortion might be recognised by the central nervous system remains unclear. The effect of changes in the amino acid balance of a diet on the brain concentrations of these

compounds, and the likelihood or otherwise of these changes being factors contributing to the depressions in food intake when diets which induce an amino acid imbalance, toxicity or antagonism are presented therefore warrants some study.

Much work has been done examining the effects of the amino acid content of the diet and of the plasma on the concentration of tryptophan and the production of 5HT in the brain. Rather less information appears to be available with regard to the catecholamines. Of particular interest is the effect of phenylalanine on catecholamine synthesis, since this amino acid is able both to reduce tyrosine uptake into the brain by competition with it for uptake across the blood-brain barrier, and to itself form a source of tyrosine within and outside the brain.

While the effect of dietary amino acid content on brain concentrations of the catecholamines and 5HT is under investigation, very little information is currently available on the effects of mineral and vitamin deficiencies on the synthesis of these neurotransmitters. The possible interaction of zinc in particular with any effect of phenylalanine on catecholamine synthesis merits some study. Wallwork, Botnen and Sandstead (1982) reported that the increased brain concentration of NE in zinc-deficient rats was not correlated with their food intake. Reeves and O'Dell (1984) however, indicated that while zinc deficiency alone had no effect on concentrations of NE and DA in the anterior hypothalamus of the rat, the reduction of the dietary tyrosine content caused a reduction in these concentrations in zinc-deficient rats which coincided with an increase in food intake. Most of the growth depression observed on feeding a zinc-deficient diet to chicks has been attributed to the accompanying reduction in food intake (Dewar, Sibbald

and Wright, 1982). It is possible therefore that the mechanism by which food intake is depressed might involve a zinc-mediated change in the brain concentrations of NE and DA.

Consideration of the phenomena of amino acid imbalances, toxicities and antagonisms is of great importance when formulating diets for feeding to animals, particularly with the increasing study of novel materials of tropical origin having potential use as feedstuffs. In addition, any elucidation of the biochemical effects of an alteration in the dietary amino acid balance could be helpful in identifying methods of improving poultry production by nutritional manipulations. Investigations of food intake patterns and changes in brain concentrations of NE, E, DA and 5HT are also potentially valuable in contributing to the knowledge of how a specific diet, alone or in combination with hormonal or drug treatment, may modify brain concentrations of the neurotransmitters, this possibly having applications in the control of disorders of the central nervous system. The current investigations were planned with the aim of studying the effect of distortions in the amino acid content and balance of the diet on the pattern of the growth and food intake responses and the brain concentrations of the catecholamines and 5HT. Broiler chicks were utilised in these investigations as sufficient numbers of the same age were easily available for large feeding experiments and the Biochemistry Department of The Edinburgh School of Agriculture was well-equipped for the handling of such birds. It had also been reported that young animals were the most sensitive to derangements in dietary amino acid balance (Harper, Benevenga and Wolheuter, 1970).

1.14 Evaluation of assay methods for neurotransmitters and metabolites

In order to obtain information on the metabolism and possible functions of the previously-mentioned neurotransmitters and some of their metabolites, assay techniques are required which are reproducible, reasonably rapid and sufficiently sensitive for use in the analysis of brain tissue. Many methods exist for the quantitation of biogenic amines, and to an extent that chosen depends on the compound of interest and the instrumentation and facilities available. Some of the procedures employed will be discused briefly.

a. Thin-layer chromatography

In thin-layer chromatography (TLC), separation of the components of a sample is brought about as each is retarded to a different extent depending on its interaction with the thin coating of stationary phase while the eluting mobile phase percolates through the plate. Many methods exist for the separation of NE, E, DA and 5HT and various metabolites by TLC (eg. Aures, Fleming and Hakanson, 1968, Osborne, 1971). An advantage of this method is that it enables the simultaneous analysis of more than one sample. Separated substances may be detected by their absorbance, fluorescence or radioactivity, or by similar properties of derivatives formed by exposing the plate to certain agents after chromatographic separation has occurred. If required, an area of the thin layer may be removed from the plate, the compound eluted from this and quantitation completed by techniques such as liquid scintillation counting or mass spectrometry. Various methods exist for the production of derivatives of the neurotransmitters and their metabolites before their separation. These may be fluorescent and aid sensitive detection, or employed to stabilise compounds such as the catecholamines, which are oxidised at alkaline pH values. Fluorigenic

detection reagents such as paraformaldehyde, ethylenediamine, (Aures *et al.*, 1968) and ortho-phthalaldehyde (OPA) (Aures *et al.*, 1968; Osborne, 1971) have been employed for the quantitation of of NE, E, DA, 5HT and various of their metabolites at the low nanogram level, other workers indicating that 1ng of 5HT was detectable by the paraformaldehyde method (Cowles, Christensen and Hilding, 1968).

While advantages exist in the availability of many detection methods and the ability to separate several samples at once, the method lacks the speed and sensitivity of some of the procedures employing high-performance liquid chromatography (HPLC), which will be discussed later. The advent of high-performance thin-layer chromatography, an improvement of the original method which employs a stationary phase of smaller particle size in a thinner, more uniform coating might perhaps give this technique greater applicability in neurotransmitter analysis. It enables a faster, more sensitive and efficient separation, using less solvent, allowing application of samples of smaller volume and a greater number of samples per plate. Because of the small sample quantities involved, analysis of separated compounds by elution methods is not feasible and quantitation is usually performed in situ by scanning densiometry.

b. Fluorescence techniques

The use of fluorescence procedures in the detection and quantitation of biogenic amines is widespread, both as a technique in itself and as the detection method in procedures such as TLC and HPLC. Native fluorescence has been used in the analysis of 5HT and 5HIAA with a detection limit of 10ng (Quay, 1963), but tends to be less sensitive and specific compared with assays employing the formation of fluorescent derivatives, since contaminating substances which might interfere in the measurement

sample and with detection methods based on an ethylenediamine or dihydroxyindole procedure, reported sensitivities of 2-5ng.

The use of fluorescence as an analytical technique requires little as regards instrumentetion and enables the assay of any compound which is itself fluorescent or capable of being converted into a fluorescent compound. However care must be taken with such assays in order to avoid error due to quenching effects, which may be caused by factors such as dissolved oxygen or the presence of certain metal ions.(Baker and Dewhurst, 1982).

c. Histochemical techniques

The localisation of NE, DA and 5HT in tissue slices by the exposure of the tissue to reagents such as formaldehyde or glyoxylic acid which produce highly fluorescent derivatives is well documented. The use of fluorescence microscopic techniques allows some degree of quantitation of the compounds within the tissue areas (Krinke and Hess, 1981). Wood (1975) employed the reaction of paraformaldehyde or glutaraldehyde-fixed material with heavy metal salts such as potassium dichromate, followed by osmium tetroxide with subsequent electron microscopy. In the absence of the dichromate both NE and E were detectable, while only NE reacted in the presence of this salt. Loren, Bjorklund, Falck and Lindvall (1976) perfused whole animals with paraformaldehyde and glyoxylic acid at acid pH and in the presence of Mg^{2+} ions, and fixed tissues with paraformaldehyde to detect nerve terminals containing NE or DA. Hess (1981) indicated that 5HT and NE were detectable in material fixed with glyoxylic acid and viewed under a fluorescence microscope at the appropriate wavelength. An alternative approach exposed the fixed tissue to monoclonal antibodies specific for 5HT, followed by a second antibody specific to the first and having a

fluorescent 'tag' attached (Consolazione, Milstain, Wright and Cuello, 1981).

Difficulties arise in the use of this technique in the routine analysis of many tissue samples. Freezing of the sample is likely to be a requirement and involves immersion in a mixture of propane and propylene cooled in liquid nitrogen so that formation of ice crystals is avoided (direct use of the nitrogen would slow cooling due to vapourisation). Freeze-drying or sectioning are followed by perfusion with the fluorogenic reagent. .The process is therefore time-consuming and fairly complex, and while it enables a very precise localisation of the amines within the structures of the brain, is not as quantitatively accurate or specific, or as versatile as other analytical methods available.

d. Gas chromatography

In this technique a sample requiring analysis is applied to an oven-heated column 1-2m in length containing an inert support material coated with a non-volatile liquid stationary phase. Components of the sample are volatilised, carried through the column by an inert gas and separated from each other due to their differential partitioning between this and the stationary phase, eluting compounds being detected in sequence as they emerge from the column. As with TLC, an advantage in the use of gas chromatography (GC) is its ability to separate and simultaneously quantify several components of a single tissue extract. Various methods of detection of eluted compounds exist. Analyses of NE and DA of rat brain by GC with a detection limit of 0.5pg were reported by Doshi and Edwards (1981) using an electron-capture detector to detect compounds derivatised in various ways before separation. However isolation of the catecholamines from the tissue extract by absorption on

alumina was necessary before processing. Martin and Ansell (1973) showed the extraction of NE, DA, DOPAC and 5HT from rat brain tissue, their derivatisation with trifluoroacetic acid and quantitation by GC with electron-capture detection at the low ng level.

While electron-capture detection is relatively non-specific, the same is not true of mass spectrometry, which may be employed as an alternative method of detection and gives high specificity and sensitivity.Koslow, Cattabeni and Costa (1972), employing pentafluoropropionic anhydride (PFPA) as the derivatising reagent, reported the measurement of NE and DA in human cerebrospinal fluid with a sensitivity of 0.5pmol (85pg and 77pg respectively), while an assay demonstrated for the dopamine metabolites DOPAC and HVA in brain tissue had a detection limit at the low nanogram level (Karoum, Gillin, Wyatt and Costa, 1975). Using the PFPA technique, 5HT was detectable in rat pineal gland at the picomole level (Cattabeni, Koslow and Costa, 1972), and in the rat brain, 5HT and its metabolite 5HIAA were both measured in a procedure with a detection limit of 10pmol (2ng) and 40pmol (8ng) (Beck, Weisel and Sedvall, 1977; Artigas and Gelpi, 1979).

Gas chromatography with either of the above methods of detection is a suitably sensitive technique for this, mass spectrometry providing the greater specificity. However in almost all cases a preliminary 'clean-up' isolation of the compounds from the original tissue extract is necessary before derivatisation and injection onto the chromatographic column. The incorporation of an internal standard which is structurally related to the compounds under investigation is also desirable in order that a correction may be made for their loss during isolation, derivatisation and separation processes. In addition, methods have been developed for the analysis of either the catecholamines and

their metabolites or for 5HT and 5HIAA, but not usually for the simultaneous assay of a selection of both types of compounds. Gas chromatography is unlikely therefore to be the method of choice for measurement of the catecholamine and indoleamine-related components of an extract of brain tissue when the processing of a large number of samples is required.

e. Radioenzymatic assay

The use of radioisotopically-labelled substrates and cofactors in the enzymic assay of neurotransmitter amines has enabled a very great increase in both the sensitivity and specificity of such analyses. In the case of a catecholamine or one of its metabolites, a radiolabelled methyl group is transferred from S-adenosyl-methionine (SAM) to the compound under investigation by the use of methyltransferase enzyme preparations such as COMT. The labelled product is isolated from the reaction mixture and its radioactivity measured.

Detection limits of 10pg for NE and DA by such a radioenzymatic assay technique employing paper chromatography for isolation of the products, were reported in 1976 by Versteeg, Van der Gugten, Dejong and Palkovits. Other assays derivatised the products before chromatographic separation (Fry, House and Sharman, 1974; McCaman, Ono and McCaman, 1979). Da Prada and Zurcher (1976), by TLC-separation of extracted o-methylated amine products and their oxidation before measurement of the radiolabel present, reported sensitivities of 1pg for NE and E, and 5pg for DA. In the rat CNS and brain regions, DA and DOPAC were estimated in assays with detection limits of 250pg and 150pg respectively (Argiolas and Fadda, 1978).

Radioenzymatic assay of 5HT was acheived by a preliminary reaction with N-acetyltransferase (NAT), the N-acetyl-5HT so formed then being converted by hydroxyindole-o-methyltransferase (HIOMT) to melatonin which was extracted and its radioactivity counted. Saavedra, Brownstein and Axelrod (1973) reported a detection limit of 50pg and high specificity for this coupled reaction, while an initial isolation of 5HT before conversion and a final TLC step enabled Boireau, Ternaux, Bourgoin, Heri, Glowinski and Hamon (1976) to measure as little as 10-20 pg 5HT in up to $3cm^3$ cerebrospinal fluid. Reader and Gauthier (1984) employed radioenzymatic assays to detect NE, E, DA and 5HT in of rat brain regions with detection limits at the low pg level.

It is apparent that radioenzymatic assay procedures are very sensitive, enabling their use for the measurement of biogenic amines in tissues such as rat brain. Derivatisations and extractions do not normally require high temperatures, so the possibility of thermal degradation which might occur with gas-chromatographic techniques is avoided. Care must be taken however that no additional enzymes present in the tissue extract are capable of affecting the levels of the compounds under assay by their action, and inhibitors of enzymes such as AADC may be added to the incubation mixture. In addition, the accuracy and reliability of the estimations depends on the efficient isolation of the radiolabelled product. When making a decision as to the appropriate method of assay, the number and identity of the neurotransmitters and metabolites under investigation, the time likely to be involved and also the financial cost of any radiolabelled substances which would be required, should be taken into consideration.

f. Other radioassays

Radioimmunoassay (RIA) employs the specific binding of antibody to antigen to estimate the amount of antigen present in a particular sample. The binding of radiolabelled antigen to a fixed quantity of antibody is partially inhibited by addition of unlabelled antigen, the extent of inhibition being related to the amount of unlabelled antigen added. Free antigen is removed by adsorption onto charcoal or anti-immunoglobulin used to precipitate the antigen-antibody complex. Bound radioactivity is counted and the amount of unlabelled antigen added estimated from calibration curves.

The small size of the catecholamine and indoleamine molecules means that it is necessary to attach them to a macromolecule in order to render them antigenic. While this conjugate is employed to produce antibodies in an appropriate animal, the antiserum so obtained will interact with the unconjugated small molecule. Choice of the conjugate must be made carefully, since modification of the smaller molecule where it attaches to the larger may result in the antiserum being unable to distinguish it from small molecules of a similar structure. Miwa, Yoshioka, Shirahata and Tamura (1977) demonstrated the production of catecholamine-bovine serum albumin conjugates and used this method in the analysis of E with a detection limit of 20pg and low cross-reactivities. COMT was used by Faraj, Walker, Camp, Ali and Cobbs (1978) to convert urinary DA to 3-methoxytyramine before the RIA of this compound, the detection limit here being 0.5ng. While 5HT was reported to be detectable at a level of 1ng in an assay devised by Peskar and Spector (1973), cross-reaction with the metabolite 5-methoxytryptamine was significant. A modification of this by Kellum and Jaffe (1976) greatly reduced this phenomenon and the assay was employed

to measure 5HT concentrations in blood and plasma samples with a detection limit of 100pg.

The great sensitivity and specificity of RIA together with its relative ease of use, make it a potentially very useful tool in the analysis of the neurotransmitter amines, Raum and Swerdloff (1981) suggesting it to be less costly and time-consuming than radioenzymatic methods. Nevertheless antisera from different animals may vary in sensitivity and the existence of cross-reactivity must also be checked. This does not appear to be one of the more popular methods of assay of the catecholamines and indoleamines in tissue samples.

g. High-performance liquid chromatography

Classical liquid chromatography involves flow of mobile phase under gravity and is often slow with poor resolution. High-performance liquid chromatography (HPLC) employs column-packing material of much smaller particle size, high flow rates achieved by pumping under pressure and a system for detection of separated components as they are eluted from the column. HPLC is normally classified into ion-exchange, normal phase and reversed-phase forms according to the packing material and process involved in the separation technique. Normal phase HPLC employs a polar packing material -normally silica -while in the reversed-phase form an organic phase is covalently bonded to the silica support. Aspects of the theory and practice of HPLC have been discussed comprehensively by Knox, Done, Fell, Gilbert, Pryde and Wall (1978) and Snyder and Kirkland (1979); and the use of bonded-phases by Majors (1980) and Goldberg (1982).

Normal phase HPLC has not been employed to a large extent for the analysis of the biogenic amines although the separation of mixtures of catecholamines or indoleamines and metabolites was

demonstrated in 1981 by Svendsen and Greibrokk. Ion-exchange HPLC was used for the separation of NE, E and DA with a sensitivity of 0.05pmol (8-9pg) by Hjemdahl, Daleskog and Kahan (1979) and 0.08pmol (13pg), 180pmol (3ng) and 200pmol (3ng) respectively by Allenmark and Hedman, (1979). Reports of the determination of DOPAC, HVA and 5HIAA at a level of less than 0.5pmol (80-100pg) (Wightman, Plotsky, Strope, Delcore and Adams (1977), and 5HT and DA at a level of 0.1pmol (15-18pg) (Sasa and Blank, 1977) by ion-exchange HPLC methods have also been made However the use of an ion-exchange column is limited in that only ionically compatible compounds may be separated and column lifetime is usually short.

Many separation techniques exist for the analysis of NE, E, DA, 5HT and various of their metabolites by reversed-phase HPLC. UV spectrophotometric detectors are not sufficiently sensitive for quantitation of such compounds at the low concentrations at which they are normally present in tissue and biological fluids, and analysis relies on fluorescence, electrochemical, or where applicable, radiochemical detection. Derivatisation of amines with OPA to give fluorescent products has been employed by Davis, Gehrke, Gehrke, Cunningham, Kuo, Gerhardt, Johnson and Williams (1979) to measure NE, DA and 5HT in plasma or urine at a detection limit of about 100pg, while measurement of DA and NE at 16pg and 5pg respectively using OPA and a laser system was acheived by Todoriki, Hayashi and Naruse (1983). Imai (1975) showed that separation and detection of 100pmol of fluorescamine-derivatised compounds was possible, however, the compounds do exhibit native fluorescence when excited at a wavelength around 285nm and this has been exploited in several reports. NE, E, DA, 5HT, and 5HIAA have been quantitated in the low ng-pg range in various combinations and with a

range of metabolites (Krstulovic and Powell, 1979; Anderson, Young, Cohen and Young, 1982; Wolf and Kuhn, 1983; Peat and Gibb, 1983). The use of fluorescence systems in HPLC has been reviewed briefly by Anderson and Young (1981), and the use of fluorigenic reagents by Imai, Toyo'oka and Miyano (1984).

Electrochemical detection (ECD)is based on the oxidation or reduction of separated components as they are eluted from the HPLC column, the fluctuation in current flow giving the required recorder signal (see Chapter Two). For the biogenic amines, the oxidative mode is usually used. In 1980, Mefford and Barchas were the first to couple reversed-phase HPLC with ECD for the detection of certain neurotransmitter amines and their metabolites in portions of rat brain, detection limits being less than 20pg. Their work has been followed by many publications applying this technique to the analysis of various groups of such compounds in samples of brain (eg. Semerdjian-Rouquier, Bossi and Scatton, 1981; Kilts, Breese and Mailman, 1981; Wagner, Vitali, Palfreyman, Zraika and Huot, 1982; Morier and Rips, 1982; Warsh, Chiu and Godse, 1982; Co, Smith and Lane, 1982; Hunt and Dalton, 1983; Mayer and Shoup, 1983). Detection limits were commonly around 200pg and in some cases direct injection of a deproteinised extract without further sample clean-up was found to give satisfactory resolution of components. The use of HPLC and ECD in the analysis of the neurotransmitter amines has been reviewed by Kissinger, Bruntlett and Shoup (1981).

Both ECD and fluorescence detection are very sensitive and accurate methods for determination of the neurotransmitter amines, and reversed-phase HPLC with either of these detectors has a number of advantages over most of the other methods discussed previously. Simultaneous separation and quantitation of several components of a

sample extract occurs within a chromatographic period of not usually more than 30-40 minutes. Thus rigorous isolation of individual components before estimation or after a derivatisation or enzymic incubation may often be avoided. In addition, the required equipment is relatively simple to operate, flexible in the range of compounds which it may be employed to separate, and allows optimisation of separations by enabling changes in mobile phase composition with time as the elution of a sample continues. ECD has been reported to be perhaps more sensitive than fluorometric techniques (Scratchley, Masoud, Stohs and Wingard, 1979), with detection limits for certain compounds under some circumstances comparable to GC with mass spectrometry (Warsh, Chiu, Li and Godse, 1980). Background noise may be kept at a minimum and a gradual loss of sensitivity avoided by occasional regeneration of the electrode surface by the appropriate method. ECD however, may however be more prone than native fluorescence to interference by oxidisable sample contaminants. While the use of reagents such as OPA could result in the production of fluorescent derivatives of contaminants as well as of the components to be assayed, the likelihood of their also fluorescing at the same wavelengths of excitation and emission as the compounds under investigation is more remote. In fact, OPA may be employed as the derivatising reagent in the quantitation of amino acids, but the derivatives so produced are excited and fluoresce in the visible region of the spectrum (eg. Umagat, Kucera and Wen, 1982). A recent development which may aid optimisation of detector sensitivity is the detection of OPA-derivatives of the neurotransmitter amines by electrochemical detection (Allison, Mayer, and Shoup, 1984).

Thus to a large extent the specificity of an analysis is dependent on the resolution of sample components achieved by the HPLC

system and the choice of detector should be influenced by the properties of the components to be analysed, perhaps with some consideration of the likely levels and properties of possible contaminants. For the neurotransmitter analyses conducted in these investigations, reversed-phase HPLC with ECD was chosen as the system of use, the reasons for this and the method employed being discussed in more detail in the following chapter.

CHAPTER TWO

EXPERIMENTAL

2.1. General Methodology

a. Source of ingredients

Maize gluten meal (Prairie Meal) was purchased from Alexander Gibson and Sons, (Blairgowrie, Perthshire) and ground oat hulls from Border Oats (Edington, Berwickshire). Ground maize, soyabean meal, fishmeal and dicalcium phosphate and calcium carbonate for mineral additions were obtained from the mill of the East of Scotland College of Agriculture (Seafield, Midlothian). Other minerals and glucose were obtained from BDH Chemicals Ltd. (Poole, Dorset). Cellulose and the purified proteins casein, gelatin and zein were purchased from the Sigma Chemical Company (Poole, Dorset). DL-methionine, the hydrochloride forms of L-lysine, L-arginine and L-histidine and the free L-forms of all other amino acids were obtained from either SAS Chemicals (London) or S+W Pharmaceuticals Ltd. (London). Their identity and purity was confirmed by the thin-layer chromatography of small samples and comparison of the chromatograms obtained with those of the known compounds. Vitamins A, E, B2, D3 and K were a gift of Roche Products Ltd. (North Dunstable, Bedfordshire) and all other vitamins were purchased from the Sigma Chemical Company (Poole, Dorset). Virginiamycin was generously provided by Smith, Kline and French (Stevenage, Hertfordshire) and 'Amprolmix' coccidiostat by the Agricultural and Food Research Council's Poultry Research Centre (Roslin, Midlothian).

b. Preparation of diets

The nutrient recommendations of the Agricultural Research Council (1975) were adopted in the formulation of the experimental diets used in the current studies. The protein content of a diet was calculated as Nx6.25, N being the dietary nitrogen content. The indispensable amino acid and nitrogen contents of maize gluten meal and fishmeal were taken

from Blair, Harber, McNab, Mitchell and Scougall (1981) and of casein from D'Mello and Emmans (1975). Similar data for soyabean meal, ground maize, gelatin and zein, and metabolisable energy data for all these mentioned ingredients were obtained from determinations previously made in the Biochemistry department of The Edinburgh School of Agriculture. The apparent metabolisable energy of oat hulls was taken from Crampton and Harris (1969), their amino acid content being low and assumed to be negligible due to poor digestibility. All these data are shown in Tables 1 and 2. Where required, the mineral and vitamin contents of feedstuffs were taken from Crampton and Harris (1969).

Ingredients for all experimental diets formulated and batches of the mixture of minerals and choline chloride were mixed with the aid of a Hobart mixer. The composition of the mixture of minerals and choline chloride is shown in Table A1. of the appendix. Vitamin mixtures were mixed manually, those vitamins requiring to be added in very small quantities being first combined in larger amounts and a proportional quantity of this mixture of 'additional vitamins' included in the final vitamin mixture. The composition of the vitamin mixture added to all diets and of the 'additional vitamins' is included in the appendix (Tables A2 and A3 respectively). The hydrochloride forms of lysine, arginine and histidine were added to diets in amounts equivalent to the quantities of the free amino acids requiring to be incorporated. Samples of the experimental diets were ground finely and submitted for analysis for dry matter, total nitrogen and gross energy contents by the Central Analytical Laboratory of the East of Scotland College of Agriculture. Estimation of total nitrogen content was by the semi-automated Kjeldahl method of Crooke and Simpson (1971), gross energy content of diets was determined by adiabatic bomb calorimetry and both values were expressed

Table 1. Content of indispensable amino acids, nitrogen and apparent metabolisable energy of ingredients employed in the current investigations

	CONTENT (g/kg dry matter)			
AMINO ACID	MAIZE GLUTEN MEAL	FISHMEAL	SOYABEAN MEAL	GROUND MAIZE
THREONINE	24.0	24.9	14.17	3.13
GLYCINE	17.4	50.6	17.77	3.22
CYSTINE	12.6	3.2	6.36	1.79
METHIONINE	24.5	15.2	5.72	1.87
LEUCINE	117.7	44.6	29.15	10.56
ISOLEUCINE	28.4	26.1	18.19	3.27
VALINE	33.0	30.7	19.20	4.21
TYROSINE	34.7	21.4	16.86	4.58
PHENYLALANINE	41.0	23.8	20.97	4.54
TRYPTOPHAN	2.6	6.9	6.00	0.90
LYSINE	10.8	48.2	23.81	2.67
HISTIDINE	14.0	14.1	11.10	2.74
ARGININE	24.1	40.5	29.26	4.49
NITROGEN CONTENT (g/kg dry matter)	96.0	104.0	70.4	14.40
APPARENT METABOLISABLE ENERGY CONTENT (MJ/kg)	16.11	11.00	9.34	14.40

Apparent metabolisable energy content of oat hulls=1.39MJ/kg. Amino acid content assumed to be negligible due to poor digestibility.

Table 2. Content of indispensable amino acids, nitrogen and apparent metabolisable energy of gelatin, zein and casein

AMINO ACID	CONTENT (g/kg) GELATIN	ZEIN	CASEIN
THREONINE	17.00	24.10	34.2
GLYCINE	201.00	9.70	18.5
CYSTINE	0.70	8.00	2.6
METHIONINE	6.00	17.30	24.5
LEUCINE	29.00	190.00	9.3
ISOLEUCINE	11.00	36.30	51.8
VALINE	21.00	35.30	69.4
TYROSINE	8.00	45.30	54.4
PHENYLALANINE	20.00	64.00	49.4
TRYPTOPHAN	0.90	2.00	9.5
LYSINE	36.00	0.70	81.9
HISTIDINE	8.00	10.70	29.6
ARGININE	74.00	13.70	36.2
NITROGEN CONTENT (g/kg)	157	134	149
APPARENT METABOLISABLE ENERGY CONTENT (MJ/kg)	17*	17	17*

*values assumed to be similar to that of zein.

on a dry matter basis, this being measured at 100°C. Where measured, the zinc content of formulated diets was determined by atomic absorption spectrometry. Alkaline hydrolysis of samples of the experimental diets enabled determination of their tryptophan content by HPLC with both electrochemical and fluorescence detection. Such analysis was carried out on specific diets by the Biochemistry department of the Edinburgh School of Agriculture.

c. Conduct of animal experiments

Day-old male [1] broiler chicks (Marshall's Ltd., Whitburn, West Lothian) were housed in [2] cages in a windowless, insulated room initially thermostatically maintained at 34°C and temperature being reduced to 29°C by the seventh day, 27°C by the fourteenth day and 24°C by the twenty-first, that is the final day of the experimental period. Light was provided for 22 hours. The chicks were initially fed a starter diet *ad lib.* (Table 4). At an age of seven days, those within a maximum weight range of 60g were then randomly assigned by computer to each cage. The computer programme was designed so as to give four chicks per cage and as small a variation in weight as possible between chicks in the same replicate, there being four replicates of each dietary treatment. A number of the birds which were no longer required were immediately decapitated and their heads frozen in liquid nitrogen to provide an estimate of brain neurotransmitter concentrations in the seven-day old chick. Experimental diets were then presented *ad lib.* to the caged chicks, the feed containers holding the stock diet and those on individual cages being weighed approximately every two days in order to determine changes in food intake occuring over the experimental period of fourteen days. The mean cumulative intake of dry matter in each case was calculated. Chicks were also weighed every two days and the mean

[1] These were a fast-growing, commercial strain, LB9.
[2] Each cage was a 37cm cube of non-galvanised stainless steel mesh, providing spaces of dimensions 1cmx2,5cm in the cage floor through which excreta fell for collection.

cumulative weight gain per chick calculated with respect to each of the diets fed.

When only four diets were to be fed, the experiment was arranged in the form of two 4x4 Latin squares and a determination of chick brain neurotransmitter concentrations was made at intervals during the feeding period. After a period of feeding appropriate to the experiment, two birds from each of the cages of one Latin square were selected using random numbers generated by a pocket calculator, weighed, decapitated and their heads immediately frozen in liquid nitrogen. The chicks remaining in the cages of this square were treated similarly some days later, and the process repeated with birds of the second Latin square at the end of the experiment, all heads being maintained at -20°C until analysis. Thus the first Latin square was used for the determination of the brain concentrations of the measured neurotransmitters in the chick at two points during the feeding period. The second remained intact throughout the experiment and was employed for measurements of chick growth and food intake over this time, and final brain concentrations of the neurotransmitters.

Any birds which died during any of the experiments conducted were sent for post-mortem analysis by the Ministry of Agriculture, Fisheries and Food Veterinary Laboratory (Lasswade, Midlothian).

d. Determination of apparent metabolisable energy

Where required, the apparent metabolisable energy content of a diet was determined and corrected for its nitrogen content, by making a collection of excreta from the chicks consuming it over the final three to four days of the feeding period. Excreta falling through the wire mesh of the cage floor was collected onto plastic sheeting placed beneath, appropriate quantities of 0.1M sulphuric acid being added daily

in order to prevent loss of nitrogen as ammonia. On the final day of the experiment, the individual excreta samples were transferred to weighed foil trays and allowed to dry completely in an oven for at least three days at 60°C. Trays containing samples were reweighed immediately upon removal from the oven and excreta samples ground in a Moulinex mill. Analysis of these for total nitrogen and gross energy contents was carried out by the Central Analytical Laboratory of the East of Scotland College of Agriculture by the previously indicated methods. Using these data and similar information obtained for the appropriate diet, together with food intake and excreta production over the collection period, values of dietary metabolisable energy corrected for nitrogen content (AME(N)) were calculated with the aid of a programme designed for use on a Commodore 'PET' microcomputer. Determinations of the dietary AME(N) content were not carried out in experiments where amino acid substitutions to the control diet were made at the expense of an isonitrogenous quantity of glutamic acid, it being considered that the resulting variation in AME(N) content would be small.

2.2. Measurement of neurotransmitters and metabolites by HPLC

a. Background

The validity of a reported method of separation of NE, E, DA, 5HT and a variety of their metabolites depends to an extent on the apparatus and materials available to individual investigator. Almost all methods of separation of compounds by HPLC require adaptation to the particular HPLC column, type of packing material and solvents employed and general working environment. When a choice of procedure for routine use is to be made, both the amount of data potentially available by use of a particular method -that is, the number of compounds able to be separated- and the reliability of the method when in frequent use must

be considered. Several methods for the separation of various groups of the neurotransmitter amines, their precursors and metabolites were therefore tested and modified before a single procedure was determined to be the most reliable, consistently giving good separation of the components of an injected mixture of compounds in a reasonably short length of time.

b. Materials

All organic solvents employed in HPLC were of highest quality, spectroscopically pure HPLC grade and supplied by Rathburn Chemicals Ltd. (Walkerburn, Peeblesshire). Sodium octyl sulfate was obtained from Eastman Kodak (Rochester, New York, U.S.A.). Norepinephrine bitartrate, epinephrine bitartrate, dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid, homovanillic acid, the creatinine sulphate salt of 5-hydroxytryptamine, the dicyclohexylammonium salt of 5-hydroxyindoleacetic acid, the disodium salt of ethylenediaminetetraacetic acid (EDTA) and o-phthalaldehyde were purchased from The Sigma Chemical Company Ltd. (Poole, Dorset). Reversed- and normal-phase 'SepPak' cartridges were obtained from Millipore Ltd. (Harrow, Middlesex), various 'Amberlite' anion and cation exchange resins from BDH Chemicals Ltd. (Poole, Dorset) and 'Norit-ol' activated charcoal from Hopkins and Williams Ltd. (Chadwell Heath, Essex). All other chemicals utilised were of 'AnalaR' grade as purchased from BDH Chemicals (Poole, Dorset).

c. Instrumentation

All analytical HPLC columns were of stainless steel, of dimensions 4.6 x 250mm and contained packing material of reversed-phase, ODS C18 type and 5μm particle diameter. This material consisted of silica particles with covalently attached hydrocarbon chains having eighteen CH2 groups. Columns were packed in the laboratory with either

'Hypersil' (Shandon Ltd., Runcorn, Cheshire) or 'Spherisorb' (Phase Separations Ltd., Queensferry, Clwyd) material using a Magnus Slurry Packer (Magnus Scientific, Cheshire). A 'Biophase' pre-packed analytical column (Bioanalytical Systems, West Lafayette, Indiana, U.S.A.) was purchased from Scotlab (Bellshill, Lanarkshire).

Samples were introduced onto the HPLC column with minimal disturbance by means of a 50µl injection syringe (Hamilton, Bonaduz, Switzerland) and a Rheodyne injection valve fitted with a 20µl sample loop. For the isocratic elution of compounds from the column, an Altex model 110-A pump was employed to pass the mobile phase through the system. A gradient of two eluting buffers was achieved when required by connection of the column to a Gilson HPLC system comprising two model 302 pumps controlled by a 'Gradient Manager' programme from an Apple II microcomputer (Scotlab). Detection of the neurotransmitters and their metabolites on elution from the analytical column was by the various methods indicated.

d. Column efficiency and phase capacity factors

Calculation of the efficiency of a column was employed to obtain an indication of the extent to which the components of a sample mixture might be separated-or resolved-by that column. It was measured as the number of theoretical plates to which the column, or a particular length of the column, was equivalent. The height equivalent to a theoretical plate, H, is defined as

$$H = \frac{1}{16} \frac{W^2}{t^2}$$

where W is the width of a peak measured in units of time and t, its retention time in the same units. The column efficiency was then equal to its length L, divided by H and was expressed as the number of theoretical plates per metre of column. The greater this number, the

greater the ability of the column to separate the components of a sample.

The capacity factor, k', is a measure of the degree of retention on the column of a particular solute. This is defined as

$$k' = \frac{t - t_o}{t_o}$$

where t_o is the time required to pass one full column volume of eluent through the column -this being the retention time of a completely unretained solute. Its value for any particular peak eluted by a specific method should be maintained even if the actual retention time shows some variation. The calculation of k' values thus enables the monitoring of the stability of a separation method during many injections of the sample mixture onto the column.

e. Standard solutions during study of separation methods

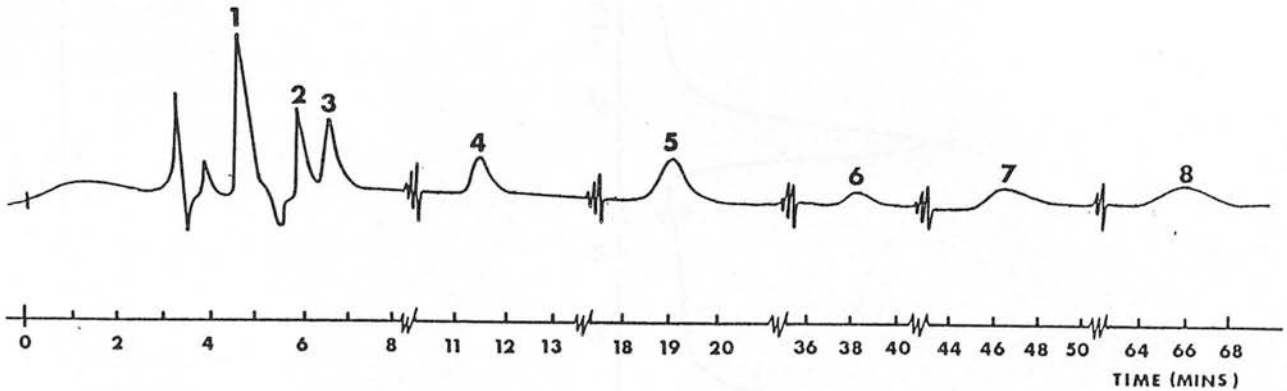
The compounds to be separated by a particular method were dissolved in 0.04M perchloric acid containing EDTA and sodium metabisulphite at concentrations of 0.1g/100cm^3 and 0.05g/100cm^3 respectively, the presence of EDTA and an antioxidant and the maintenance of all solutions at 4°C aiding sample stability (Verbiese-Genard, Hanocq, Alvoet and Molle, 1983). The concentrations of the solutions depended on the form of detection employed. In the case of the u/v spectrophotometric detector which measured absorbance of light at 280nm, concentrations were of the order of 5mg/cm^3 or 100ng per 20µl injected; for the fluorometer and electrochemical detector subsequently used, solutions were at a concentration of 50ng/cm^3, or 1ng per 20µl injected.

f. Selection of separation method

i. Initial procedures

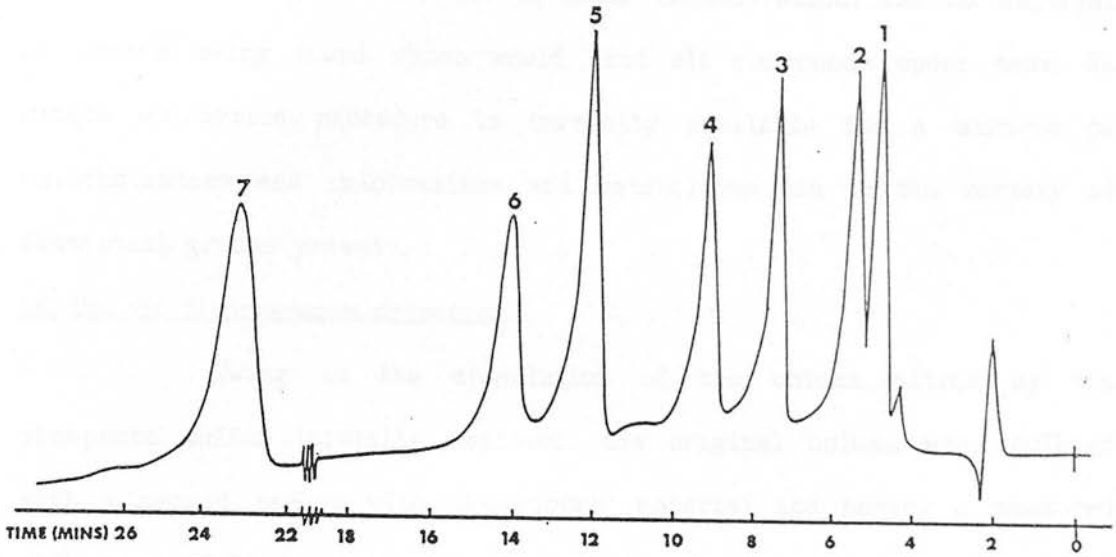
At the outset, development of a method of separation of NE, E, DA, 5HT and some of their metabolites employed an Hitachi model 100-10 u/v spectrophotometric detector set at a wavelength of 280nm and an analytical column packed in the laboratory with 'Hypersil' material and having a measured column efficiency of approximately 40000 plates per metre. At a flow rate of $0.7cm^3min^{-1}$, a mobile phase of 0.1M disodium phosphate/citric acid buffer pH 4.7 as employed by Semerdjian-Rouquier, Bossi and Scatton (1981) but incorporating 1.5% (v/v) acetonitrile rather than 5% (v/v) methanol, achieved the separation of NE, E, DOPA, DA, 5HTP, 5HT, tryptophan and 5HIAA. A chromatogram of a standard mixture of these is shown in Fig. 6. The long period required for this separation however was unsatisfactory and the method of Mayer and Shoup (1983) was subsequently adopted, the eluting buffer consisting of 3.5 parts acetonitrile, 1.8 parts tetrahydrofuran and 96.5 parts of 0.15M monochloroacetic acid pH 3.0 containing sodium octyl sulphate at a concentration of 0.86mM. This enabled reasonably good resolution of the components of a standard mixture of NE, E, DOPAC, DA, 5HIAA, homovanillic acid (HVA) and 5HT in approximately twenty-five minutes at a flow rate of $1.2cm^3min^{-1}$ (Fig. 7), somewhat less than the reported flow rate of $1.6cm^3min^{-1}$.

Although the use of u/v spectrophotometric detection was adequate during the study of separation methods, such instrumentation was not sufficiently sensitive for the detection and analysis of amine neurotransmitters and metabolites in actual extracts of the chick brain. Attempts were made to find a rapid way to concentrate and 'clean up' deproteinised brain extracts. Such extracts or standard mixtures of the



PEAK	K'
1. NE	0.42
2. E	1.00
3. DOPA	0.82
4. DA	2.48
5. 5HTP	4.79
6. 5HT	10.61
7. Tryptophan	13.15
8. 5HIAA	19.03

Fig. 6. Chromatogram of a mixture of eight neurotransmitters, metabolites and precursors as acheived by a modification of the method of Semerdjian-Rouquier, Bossi and Scatton (1981). Conditions as in text.



PEAK	K'
1. NE	1.3
2. E	1.65
3. DOPAC	2.55
4. DA	3.40
5. SHIAA	4.35
6. HVA	5.90
7. SHT	10.55

Fig. 7. Chromatogram of a mixture of seven neurotransmitters and metabolites, obtained by a modification of the method of Mayer and Shoup (1983). Conditions as in text.

amines and metabolites in 0.4M perchloric acid or 80% (v/v) ethanol in water were passed through reversed- and normal-phase 'SepPak' cartridges, small amounts of various 'Amberlite' anion or cation exchange resins or 'Norit-ol' activated charcoal. No benefit was observed as regards aiding detection at 280nm, elution of compounds where binding did occur allowing no increase in their concentration, and no material or method being found which would bind all compounds under test. No single extraction procedure is currently available for a mixture of catecholamines and indoleamines and metabolites due to the variety of functional groups present.

ii. Use of fluorescence detection

Owing to the dissolution of the column silica by the phosphate buffer initially employed, the original column was replaced with a second packed with 'Spherisorb' material and having a measured efficiency of 24000 plates per metre. This was connected to a Gilson model 121 fluorometer having an excitation wavelength of 340nm and emission wavelength of 455nm (purchased from Scotlab, Bellshill, Lanarkshire). According to information supplied by the manufacturers, the neurotransmitter amines should have been detectable by their native fluorescence when excited by this latter instrument, but these studies failed to confirm this. The fluorometer gave excitation in the visible wavelength only and no response was obtainable on injection of the standard mixture into the system. Other reports of detection by native fluorescence have utilised excitation in the u/v wavelengths (Krstulovic and Powell, 1983; Peat and Gibb, 1983). Derivatisation of a mixture of amines and certain metabolites by o-phthalaldehyde was carried out according to the method of Davis *et al.* (1979) in order that a new

method for the separation of the fluorescent derivatives might be developed; however due to instrument failure this could not be pursued.

iii. Electrochemical detection

It was decided that for the determination of neurotransmitters such as 5HT and the catecholamines in the brain of the chick, an electrochemical detector was the most suitable instrument of detection, since the compounds of interest were detectable by this in the picogram range without any necessity for the production of reactive derivatives. The instrumentation employed consisted of an LC17 electrochemical transducer cell and LC3A controller (Bioanalytical Systems, West Lafayette, Indiana, U.S.A.; as purchased from Scotlab). A working electrode potential of 0.72V versus an Ag/AgCl reference electrode was employed. It was decided to study a separation based on the report of Hunt and Dalton (1983) employing a non-linear gradient of 1-20% (v/v) acetonitrile in potassium phosphate buffer pH3.5 containing 4mM sodium heptanesulphonate and 100μM EDTA. A modification of the gradient allowed the separation of NE, E, DOPA, DOPAC, 5HTP, DA, 5HIAA, HVA and 5HT as they had reported, this being an improvement over the separation of only seven of these compounds by Mayer and Shoup (1983). Reasonable separation of some of the compounds nevertheless required a considerably longer run time than had been reported and a continual shifting in the retention times of eluted compounds was found (Fig. 8), probably due to gradual evaporation of acetonitrile from the eluent. As complete sealing of the gradient system was impossible it was decided to return to the previously-employed isocratic system of Mayer and Shoup (1983), with the use of a pre-packed 'Biophase' column having an efficiency of approximately 17000 plates per metre. Possible evaporation of solvents incorporated into the eluting buffer could be avoided by

PEAK	K'(a)	K'(b)
1. DOPA	4.67	2.56
2. NE	4.87	2.78
3. E	8.73	6.99
4. DOPAC	11.05	10.73
5. 5HTP	12.35	11.71
6. DA	14.30	13.98
7. 5HIAA	15.03	14.71
8. HVA	15.95	15.77
9. 5HT	17.78	17.60

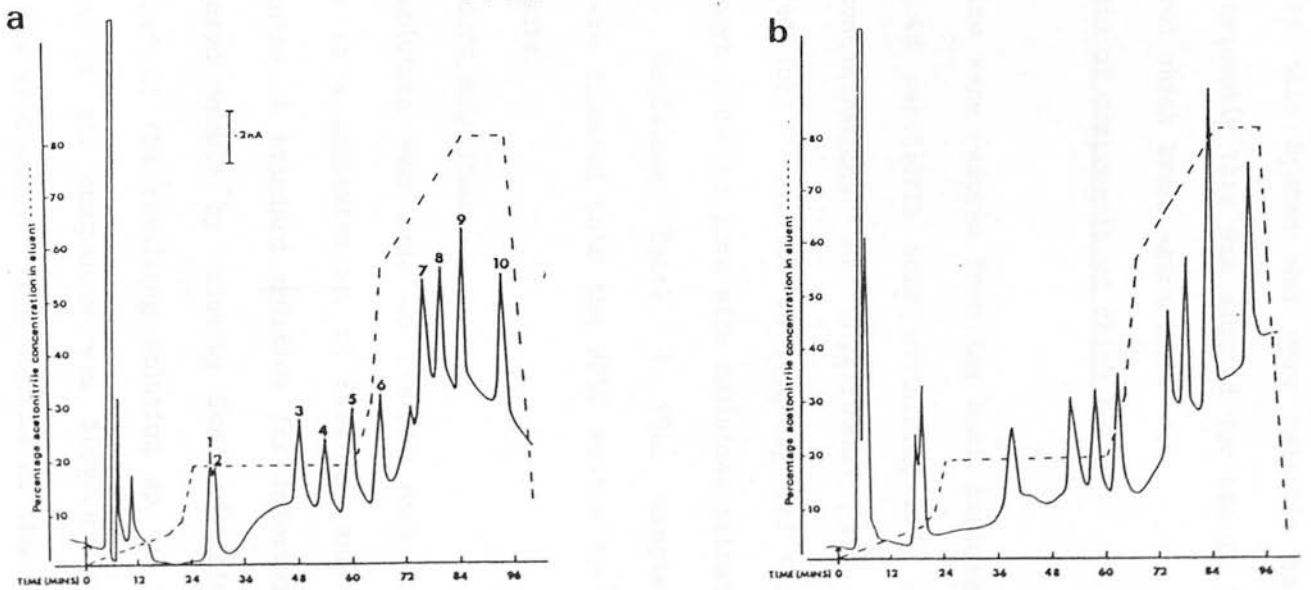


Fig. 8. Comparison of two chromatograms obtained for a standard solution of nine neurotransmitter amines, precursors and metabolites, under apparently identical gradient elution conditions, modified from Hunt and Dalton (1983). Conditions as in text.

recycling of the column effluent into the buffer reservoir and complete sealing of the system (R. Talbot, AFRC Institute of Animal Physiology and Genetics Research, Roslin, Midlothian; personal communication). The separation of a mixture of seven neurotransmitter amines and their metabolites achieved by this system was very reliable and occurred within 30 minutes, consequently this was adopted for use in the routine analysis of deproteinised chick brain extracts.

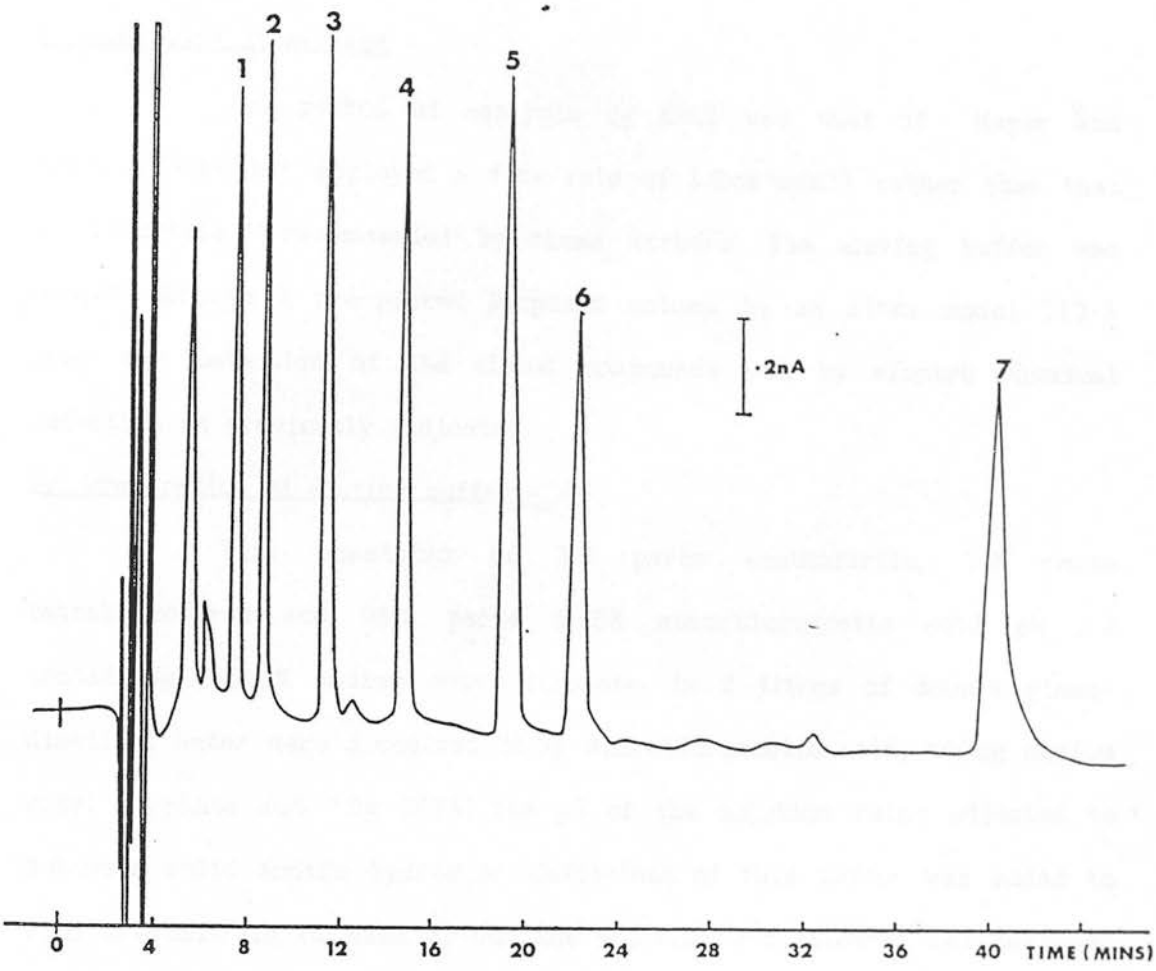
g. Procedure for analysis of components of chick brain

i. Sample preparation

Chick brains were removed from the head, homogenised in 10 volumes of ice-cold 0.4M perchloric acid containing EDTA and sodium metabisulphite at concentrations of 0.1g/100cm^3 and 0.05g/cm^3 respectively, centrifuged for 15 minutes at 10,000xg and the resulting supernatant passed through a 0.45μm pore size cellulose nitrate membrane filter (Whatman Ltd., Maidstone, Kent). A 20μl sample of this deproteinised extract was injected onto the HPLC system for resolution of the relevant components.

ii. Preparation of standard solutions

A stock solution was made to contain each of the free amines and metabolites at a concentration of 50μg/cm^3 and was stable for at least three months. A standard solution for injection into the HPLC system was prepared weekly by diluting 5cm^3 of this stock to 500cm^3 and making 10cm^3 of the resulting solution up to 100cm^3. The resulting concentration of all compounds was 50ng/cm^3 or 1ng per injection of 20μl onto the HPLC column. The response of the detector was linear to this amount, the limit of detection being approximately three times the baseline noise-approximately 30pg for all compounds but HVA, the detection limit of this being approximately 60pg. A chromatogram of



PEAK	K'
1. NE	1.55
2. E	1.90
3. DOPAC	3.00
4. DA	4.10
5. 5HIAA	5.69
6. HVA	6.72
7. 5HT	15.18

Fig. 9. Chromatogram of a standard mixture of amines and metabolites by a slight modification of the method of Mayer and Shoup (1983) and using electrochemical detection. Conditions as in text.

the standard mixture is shown in Fig. 9. All solutions were refrigerated when not in use.

iii. Analysis of extract

The method of analysis by HPLC was that of Mayer and Shoup (1983), but employed a flow rate of 1.2cm^3min^{-1} rather than that of 1.6cm^3min^{-1} recommended by these workers. The eluting buffer was passed through a pre-packed Biophase column by an Altex model 110-A pump and detection of the eluted compounds was by electro chemical detection as previously indicated.

iv. Preparation of eluting buffer

This consisted of 3.5 parts acetonitrile, 1.8 parts tetrahydrofuran and 96.5 parts 0.15M monochloroacetic acid pH 3.0 containing 0.86mM sodium octyl sulphate. In 2 litres of double glass-distilled water were dissolved 28.3g monochloroacetic acid, 400mg sodium octyl sulphate and 0.5g EDTA, the pH of the solution being adjusted to 3.0 with solid sodium hydroxide. Sufficient of this buffer was added to 70ml acetonitrile to make 2l and the solution was filtered and degassed by filtration under vacuum through a 0.7μm glass fibre filter (Whatman Ltd., Maidstone, Kent), followed by a very brief period in an ultrasonic bath. 36ml of tetrahydrofuran was then very gently mixed in, the container sealed tightly and stored at 4°C, being allowed to reach room temperature before use.

v. Working of the electrochemical detector

A cross-sectional diagram of the electrochemical transducer cell employed is shown in Fig. 10. A PTFE gasket of thickness 125μm is tightly sandwiched between two plastic blocks to leave a rectangular channel within, the planar glassy carbon working electrode being embedded in the upper surface of the lower block so as to lie in the

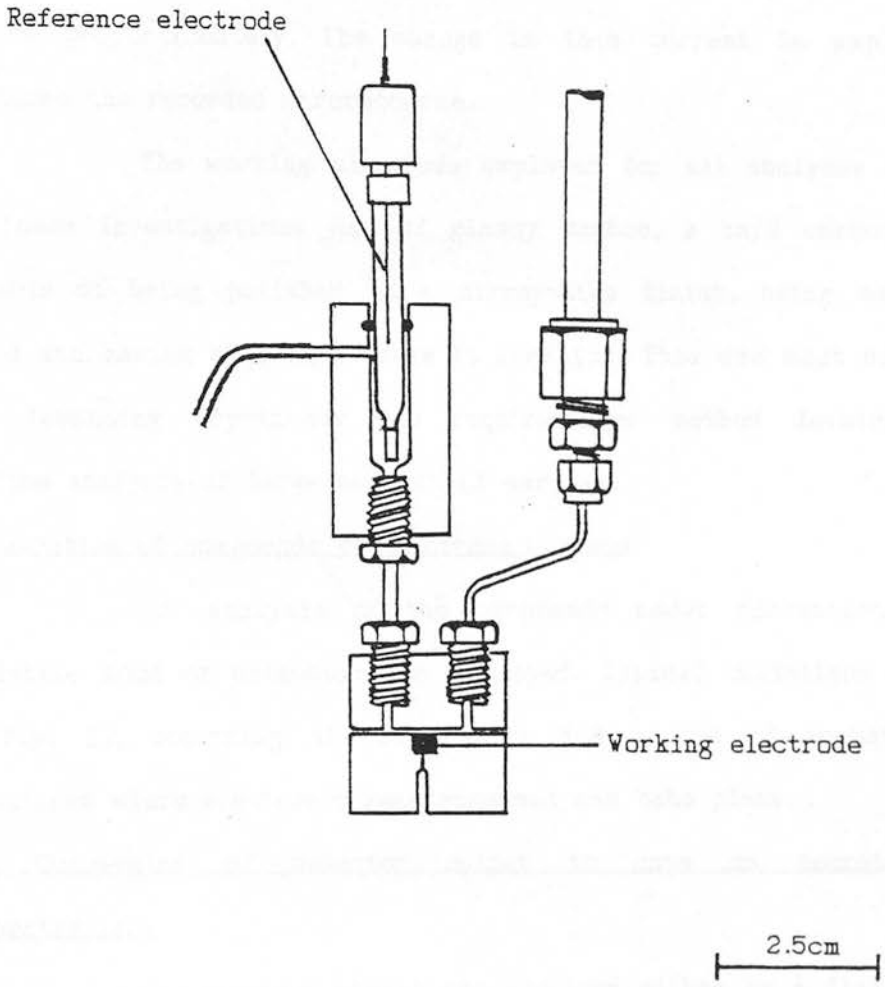


Fig. 10. Cross section of an LC17 Bioanalytical Systems electrochemical transducer cell
From LC17/LC3A operating manual, 1980. Bioanalytical Systems, West Lafayette, Indiana, U.S.A.

floor of this channel. Effluent from the column passes as a thin film across this electrode and subsequently over the Ag/AgCl reference electrode before exiting from the instrument. The working electrode is held at a fixed potential relative to the reference electrode and if this is sufficiently greater (or smaller) than that required for electrolysis of solute molecules passing over it, then that solute will be oxidised (or reduced). As the concentration of a responsive solute alters within the mobile phase flowing over the electrode, the electrolysis current alters proportionately. The change in this current is amplified and produces the recorded chromatogram.

The working electrode employed for all analyses undertaken in these investigations was of glassy carbon, a hard carbon material capable of being polished to a mirror-like finish, being mechanically rigid and having high resistance to organics. This was most suitable for the demanding day-to-day use required for method development and routine analysis of large numbers of samples.

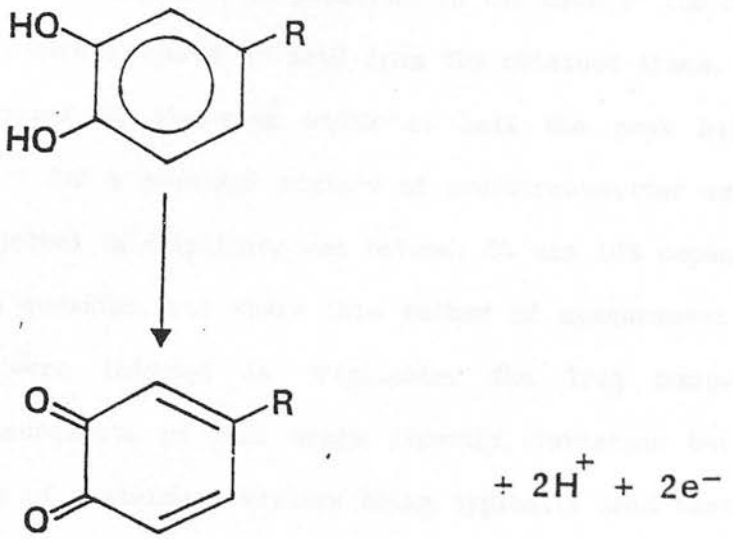
vi. Reaction of compounds at electrode surface

For analysis of the compounds under discussion here the oxidative mode of detection was employed. Typical oxidations are shown in Fig. 11, occurring at the O-H or N-H groups of aromatic cyclic structures where electronic rearrangement can take place.

vii. Conversion of detector output to data on neurotransmitter concentrations

The detector signal was received either by a flat-bed chart recorder having a full-scale deflection of 1mV (Linseis, purchased from Scotlab, Bellshill, Lanarkshire) or a Trio computing integrator (Trivector Systems International, Bedfordshire). In each case concentrations of neurotransmitters or their metabolites in brain

i.



ii.

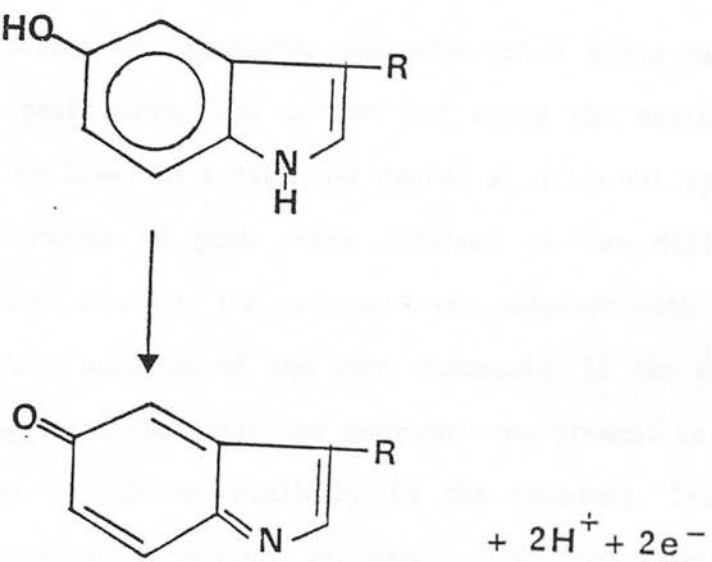


Fig. 11. Two electron mechanism of oxidation of i. catecholamines and ii. indoleamines

extracts were determined by comparison of the obtained peak areas with those given on injection of known amounts of an external standard mixture of the compounds under analysis, recoveries being measured as 97-110%, depending on the compound in question. In the case of the chart recorder, peak areas were measured by hand from the obtained trace, peak height being multiplied by the peak width at half the peak height. Intra-assay variation for a standard mixture of neurotransmitter amines and metabolites injected in triplicate was between 5% and 10% depending on the substance in question, and where this method of measurement was employed, samples were injected in triplicate. The Trio computing integrator gave measurements of peak areas directly, variation between triplicate injections of a standard mixture being typically less than 5%. Measurements were subsequently made on single injections of extract samples.

viii. Identification of eluted peaks

Identification of the eluted compounds of a brain extract and confirmation of peak purity was carried out using the ability of each compound to be oxidised to a different degree at different applied potentials. Response ratios of peak areas obtained at two different applied potentials were calculated for each peak and compared with those obtained for a standard solution of the pure compounds. If the ratios were similar this suggested that only one compound was present in each eluted peak and that it behaved similarly to the standard. Table 3 illustrates the response ratios obtained for peaks eluted from injections of standards and samples at potentials of 0.60V and 0.72V. Such measurements were made at intervals throughout the analyses. Otherwise, retention times and relative retention times ($\hat{k'}$) were employed to identify the components of interest in an injected sample.

Table 3. Response ratios (peak area at 0.72V/peak area at 0.6V) of compounds detected in both standard mixture and chick brain extract.

	Ratio	
Compound	Standard	Extract
NE	1.48	1.47
E	1.60	1.62
DA	2.13	2.01
DOPAC	1.38	1.48
HVA	1.17	1.20
5HIAA	10.91	10.55
5HT	1.06	1.12

2.3. Experiments conducted

a. Experiment 1. Feeding of the starter diet

Aims

A starter diet (Table 4), was fed to chicks in all investigations, experimental diets being presented after the birds reached an age of seven days. It was determined to allow a group of chicks to continue to receive this starter diet over what would usually be the experimental period, in order to estimate the possibility of diurnal variation causing differences between the brain neurotransmitter concentrations of chicks killed at different times of the day. Some evidence of diurnal variations in the brain concentrations of 5HT and 5HIAA in the rat has been reported by Fernstrom *et al.* (1985).

Procedure

Chicks were fed the starter diet to an age of seven days as previously indicated. Four chicks in each of eight cages continued to be fed the starter diet and after a further fourteen days half of these were killed and heads collected, one chick from each of four cages being killed between 09.50h and 10.00h, two chicks from a second four cages between 13.55h and 14.15h, and one chick from each of the four original cages between 15.50h and 16.00h. Seven days later, chicks remaining in the first four cages were killed between 09.50h and 10.00h and the others between 14.00h and 14.20h. Patterns of growth and food intake were recorded throughout.

b. Experiment 2. The lysine-arginine antagonism

Aims

The antagonism of arginine by an excess of dietary lysine is well known, many studies of this phenomenon having been made in poultry (Jones, 1964; Boorman and Fisher, 1965; O'Dell and Savage, 1966;

Table 4. Composition of starter diet and of control diets employed in experiments 2-5

Content of ingredients

	AMOUNT INCORPORATED (g/kg diet)				
INGREDIENTS	STARTER	EXPT.2	EXPT.3	EXPT.4	EXPT.5
GROUND MAIZE	609.42	691.84	575.50	775.10	20.30
SOYABEAN MEAL	243.00	106.05	300.00		112.40
FISHMEAL	100.00				
OAT HULLS					24.59
MAIZE GLUTEN MEAL					91.20
CASEIN		35.00			
ZEIN		30.00			
GELATIN				100.00	90.80
GLUCOSE				51.80	581.60
$MgSO_4.7H_2O$					1.01
$KHCO_3$					1.33
MINERALS+CHOLINE[a]	44.30	44.30	44.30	44.30	44.30
VITAMIN MIX[b]	0.60	0.60	0.60	0.60	0.60
MAIZE OIL		2.51			10.00
AMINO ACID MIX A[c]				28.20	
AMINO ACID MIX B[c]					20.30
L-GLUTAMIC ACID		80.00	73.10		1.57
L-THREONINE			2.00		
L-LYSINE.HCl		4.50	2.50		
L-TRYPTOPHAN		0.60			
DL-METHIONINE	2.68	4.60	2.00		
	1000.00	1000.00	1000.00	1000.00	1000.00

[a]Table A1, appendix. [b]Table A2, appendix. [c]Table 5.

Analytical composition

NITROGEN CONTENT (g/kg dry matter)	42.95	34.62	35.82	34.76	38.70
AME(N) CONTENT (MJ/kg dry matter)	13.95	14.00[d]	13.61[e]	14.22[e]	13.46

[d]D'Mello, J.P.F. and Borras, F. unpublished data.
[e]D'Mello, J.P.F. unpublished data.

Amino acid content (g/kg dry matter)

THREONINE	9.87	6.09	8.94	7.42	7.76
GLYCINE	13.13	5.51	7.98	25.26	22.99
CYSTINE	3.87	2.47	3.25	3.24	2.07
METHIONINE	7.46	8.63	5.33	7.94	7.61
LEUCINE	19.46	21.17	16.46	12.49	17.69
ISOLEUCINE	9.84	7.77	8.15	8.04	8.93
VALINE	11.80	9.23	9.09	8.27	10.36
TYROSINE	9.96	9.00	8.54	4.91	6.17
PHENYLALANINE	11.56	9.83	9.89	14.03	10.40
TRYPTOPHAN	2,89	2.46	2.58	0.89	1.06
LYSINE	11.69	11.89	11.86	12.00	11.61
HISTIDINE	5.94	4.82	5.45	4.93	5.03
ARGININE	14.92	8.64	12.62	12.23	12.91

Table 5. Composition of amino acid mixtures A and B incorporated into control diets of experiments 4 and 5 respectively

AMINO ACID	MIX A.	MIX B.
L-THREONINE	2.50	2.00
DL-METHIONINE	5.00	3.80
L-ISOLEUCINE	3.50	2.80
L-VALINE	2.00	2.70
L-PHENYLALANINE	7.00	1.90
L-LYSINE.HCl	6.30	5.10
L-HISTIDINE.HCl	1.90	2.00
	28.20	20.30

Smith and Lewis, 1966; D'Mello and Lewis, 1970a; Kadirvel, Vohra and Kratzer, 1974; Wilburn and Fuller, 1975). There have been no data published however, on the effects which the depressions in growth and food intake caused by excessive dietary lysine, and the restoration of these measurements by supplemental arginine, might have on brain neurotransmitter concentrations. A classical lysine-arginine antagonism was therefore induced in order to provide information on this point.

Procedure

To a control diet formulated so as to be slightly deficient in arginine, was added a small supplement of arginine of 3.5g/kg, an excess of lysine at a level of 15g/kg, or excess lysine combined with an arginine supplement of 12g/kg. All additions were made at the expense of an isonitrogenous amount of glutamic acid. The experiment was arranged in two 4x4 Latin squares. Heads were collected from two chicks from each cage of one square after four and after eight days of feeding the diets. Changes in weight gain and food intake were calculated for chicks of the remaining intact square, which were treated similarly on the final day of the experiment. The composition of the control diet is shown in Table 4.

c. Experiment 3. The effect of supplementary glycine and arginine on methionine toxicity

Aims

Methionine is one of the most toxic amino acids when fed in excess, and a possible alleviation of its effects in the rat by glycine and arginine has been reported (Waterhouse and Scott, 1961; Benevenga and Harper, 1967; Smith, 1969b). As methionine is transported across the blood-brain barrier by the same amino acid carrier system that is responsible for transport of tryptophan, tyrosine and the other LNAA,

the possibility existed that a high concentration of methionine might compete significantly with the precursor amino acids and thus affect brain concentrations of NE, DA and 5HT. The phenomenon was therefore investigated in the chick

Procedure

To a control diet adequate in all indispensable nutrients (Table 4), was added methionine at a concentration of 2g/kg either alone, with glycine at 1g/kg, or with glycine and arginine each incorporated at a concentration of 1g/kg. All of these additions were again made at the expense of an isonitrogenous quantity of glutamic acid. The experiment was designed in the form of two 4x4 Latin squares, chicks of one square being killed and heads collected at two points during feeding and those of the other at the end of the experimental period.

d. Experiment 4. The induction of a dietary tryptophan imbalance

Aims

The adverse effects of feeding poultry diets which are imbalanced have been demonstrated widely (Anderson *et al.*, 1951; Hill and Olsen, 1963; Davis and Austic, 1982a). An amino acid imbalance with respect to tryptophan was considered to be of particular interest, as this indispensable amino acid was the precursor of 5HT, synthesis of which appeared to be largely dependent on the concentration of tryptophan in the brain (Chapter 1). Since the brain tryptophan concentration seemed to depend on the relative plasma concentrations of this amino acid and its competitors for uptake across the blood-brain barrier, inducing a relative deficiency of tryptophan might be expected to influence 5HT synthesis.

Procedure

A diet was formulated so as to be low in tryptophan while adequate in all other dietary components. This was fed alone and also with the addition of a supplement of tryptophan of 1.5g/kg. In addition, the effect of incorporation of a mixture of all indispensable amino acids except tryptophan, at a total concentration of 39g/kg was investigated in the absence and presence of the tryptophan supplement. The composition of the control diet is shown in Table 4, and that of the mixture incorporated as part of it to ensure an adequate amino acid content, in Table 5. The composition of the indispensable amino acid mixture added in order to induce an amino acid imbalance is shown in Table 6. Additions of amino acids to the control diet were at the expense of an equal weight of glucose, since maintenance of the dietary nitrogen level by making substitutions for an isonitrogenous quantity of glutamic acid would have required too high a level of incorporation of this latter amino acid into the control diet. The experiment was again arranged as two 4x4 Latin squares, birds of one square being killed at intervals as previously described, and the others at the end of the feeding period.

e. Experiment 5. Amino acid imbalance and responses to tryptophan supplementation

Aims

There are currently two methods employed for the empirical estimation of the amino acid requirements of poultry. In a 'graded supplementation' technique (eg. Boomgardt and Baker, 1973), a diet is composed in which the amino acid under test is first-limiting for growth according to available estimates of its requirements. This is fed alone and with increasing amounts of the amino acid under investigation,

Table 6. Indispensable amino acid mixture added to control diet of experiment 4. in order to create an amino acid imbalance with respect to tryptophan

Based on Sanahuja and Harper (1963b)

AMINO ACID	AMOUNT ADDED (g/kg diet)
L-THREONINE	4.00
DL-METHIONINE	4.00
L-LEUCINE	6.00
L-ISOLEUCINE	4.00
L-VALINE	4.00
L-PHENYLALANINE	6.00
L-LYSINE.HCl	6.00
L-HISTIDINE.HCl	2.00
L-ARGININE.HCl	3.00
	39.00

and the growth response as measured after some weeks of feeding is used to produce a curve from which the amino acid's requirement may be estimated. It has however been argued that the alteration in amino acid balance of successive diets may influence the results obtained and that at high levels of supplementation the amino acid under test may no longer be limiting for growth (Fisher and Morris, 1970).

An alternative method of estimation of amino acid requirements was therefore proposed (Fisher and Morris, 1970). In a 'diet dilution' method, a so-called 'summit' diet is composed so as to include all indispensable amino acids apart from that under test at a high level, e.g. 180% of their requirements. That under test is at a lower level, e.g. 140% of its currently estimated requirement. The summit diet is then diluted with an isoenergetic protein-free mixture to produce a series of diets having the same relative amino acid balance yet having different concentrations of the amino acid under test, which is limiting in all cases. A growth response is taken as a response to changes in the concentration of this amino acid and its requirement estimated from a curve as before. However it is apparent that protein concentrations of the diets are also altered, and in addition the diets have been deliberately formulated so as to be imbalanced, that is, having a relative deficiency of one amino acid. Such distortion of amino acid balance could also be argued to influence the results obtained. In fact, each estimation technique gives a similar result and their relative merits have been discussed (D'Mello, 1982). However, the patterns of growth and intake over the feeding period may not necessarily be the same in each case and it was determined therefore to make a comparison of the two techniques with respect to the estimation of the tryptophan

requirement of the chick and accompanying differences in the chick brain concentrations of 5HT. .

Procedure

The tryptophan-limiting diet employed for the graded supplementation method is shown in Table 4. Due to its highly purified nature, supplements of potassium and magnesium salts were incorporated in addition to those supplied by the minerals+choline mixture, together with small amounts of nicotinic acid, calcium pantothenate, pyridoxine hydrochloride, vitamin B_{12} and biotin. The amino acid mixture added to ensure adequate dietary concentrations of all indispensable amino acids but tryptophan is shown in Table 5. Three different amounts of tryptophan were added to the control diet at the expense of an isonitrogenous quantity of glutamic acid, giving four diets in all.

The composition of the summit diet and the dilution mixture employed for the diet dilution method of estimating amino acid requirements are shown in Table 7. These were of a similar calculated AME per kg fresh diet to that of the diets used in the graded supplementation method and dilutions of the summit were arranged so as to give diets with identical tryptophan concentrations to those produced by that method (Table 8). Due to the high level of dilution required for the diet containing the lowest concentration of tryptophan, an additional supplement of potassium (as $KHCO_3$) was incorporated into both summit and dilution mixtures, together with the same extra vitamins as had been added to those diets containing graded supplements of tryptophan. The experiment was conducted in a randomised block arrangement and chicks killed and heads collected at the end of the experimental period.

Table 7. Composition of summit diet and dilution mixture employed in the estimation of the tryptophan requirement of the chick by the 'diet dilution' technique (Experiment 5).

Summit diet
Content

INGREDIENT	AMOUNT INCORPORATED (g/kg diet)
GROUND MAIZE	224.40
SOYABEAN MEAL	338.00
MAIZE GLUTEN MEAL	273.70
GELATIN	100.00
MINERALS+CHOLINE	45.10
VITAMINS	0.60
DL-METHIONINE	1.20
L-LYSINE.HCl	7.00
MAIZE OIL	10.00
	1000.00

Analytical composition

TOTAL NITROGEN CONTENT (g/kg dry matter)	69.77
ESTIMATED AME PER kg FRESH MATTER (MJ)	12.87
ESTIMATED AME per kg DRY MATTER (MJ)	14.08

Amino acid content (g/kg dry matter)

THREONINE	15.06
GLYCINE	34.57
CYSTINE	6.65
METHIONINE	11.89
LEUCINE	51.77
ISOLEUCINE	17.24
VALINE	20.30
TYROSINE	18.64
PHENYLALANINE	23.33
TRYPTOPHAN	3.31
LYSINE	22.76
HISTIDINE	9.83
ARGININE	27.25

Dilution mixture

INGREDIENT	AMOUNT INCORPORATED (g/kg diet)
CORNSTARCH	300.80
GLUCOSE	555.20
OAT HULLS	88.30
MINERALS+CHOLINE	45.10
VITAMIN MIX	0.60
MAIZE OIL	10.00
	1000.00

ESTIMATED AME CONTENT PER kg FRESH MATTER (MJ)	12.87
ESTIMATED AME CONTENT PER kg DRY MATTER (MJ)	13.41

Table 8. Proportions of summit diet and dilution mixture combined to produce diets containing varying concentrations of tryptophan

EXPERIMENTAL DIET	AMOUNTS COMBINED (Kg) SUMMIT	DILUTION MIXTURE	TRYPTOPHAN CONTENT AS PROPORTION OF ASSUMED REQUIREMENT
V	3.333	6.667	0.48
VI	4.348	5.652	0.63
VII	5.435	4.565	0.78
VIII	6.944	3.056	1.00

Table 11. Mixture of indispensable amino acids added to control diet of experiment 6 in order to create an amino acid imbalance with respect to tyrosine and phenylalanine

AMINO ACID	AMOUNT ADDED (g/kg diet)
L-THREONINE	7.40
L-GLYCINE	14.00
DL-METHIONINE	9.20
L-LEUCINE	14.70
L-ISOLEUCINE	8.50
L-VALINE	9.80
L-TRYPTOPHAN	2.10
L-LYSINE.HCl	13.80
L-HISTIDINE.HCl	6.00
L-ARGININE.HCl	12.40
	97.90

at about half their required total concentration. The composition of this diet is shown in Table 9. Due to its highly purified nature, additional supplementation with certain vitamins and potassium (as $KHCO_3$) over those amounts added in the minerals+choline and vitamin mixtures was necessary. In order to produce an adequate dietary content of all indispensable amino acids but the catecholamine precursors, the control diet also included a mixture of amino acids, the composition of which is shown in Table 10. A second diet was created by the addition to this control diet of a crystalline mixture of all indispensable amino acids except tyrosine and phenylalanine at concentrations equal to their requirements. This addition was deemed to be the method most likely to produce an imbalance with respect to the catecholamine precursors. In order to alleviate the consequent effects, further diets included additional phenylalanine at a concentration of either 5g/kg or 10g/kg. All amino acid additions to the control diet were made at the expense of glucose, as it would have been necessary to include an excessive amount of glutamic acid into the control diet in order that isonitrogenous substitutions might be made. Composition of the mixture of amino acids added to create the imbalance is shown in Table 11. This mixture raised the calculated dietary crude protein content from 120g/kg in the control diet to 220g/kg in the others, additional phenylalanine supplementation having little effect.

The four diets thus produced were zinc-deficient. A second group was formulated similarly, except that zinc acetate was now incorporated into the minerals+choline mixture so as to increase dietary zinc content by approximately 50mg/kg. This created zinc-adequate versions of the four diets first mixed. All chicks received deionised water throughout the experiment in order to eliminate all non-dietary

Table 9. Composition of control diets employed in experiments 6-13

	AMOUNT INCORPORATED (g/kg diet)					
INGREDIENT	EXPTS. 6/7	EXPT. 8	EXPT. 9	EXPTS. 10/11	EXPT. 12	EXPT. 13
GROUND MAIZE	500.00	219.40	597.80	300.00	600.00	742.30
SOYABEAN MEAL		290.00	183.20	207.60	50.00	
FISHMEAL		90.00		100.00	25.00	
GROUND OAT HULLS				20.00		
ZEIN					56.00	7.70
GELATIN	30.00		12.00	27.00	31.00	
GLUCOSE	364.00	342.20		256.40	113.20	
CORNSTARCH					76.00	
MINERALS+CHOLINE[a]	44.30	44.30	44.30	44.30	44.30	44.30
VITAMIN MIX[b]	0.60	0.60	0.60	0.60	0.60	0.60
$KHCO_3$	2.70					1.50
L-GLUTAMIC ACID			101.20		41.40	61.10
MAIZE OIL	10.00	10.00	46.00	40.00	20.00	10.00
DL-METHIONINE		3.50		3.60		
L-THREONINE				0.50		
AMINO ACID MIX C[c]	48.40					
AMINO ACID MIX D[c]			14.90			
AMINO ACID MIX E[c]					18.50	
	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

[a]For composition see Appendix, Table A1. That added to control diet of experiment 7 was formulated without the addition of zinc acetate.

[b]Appendix, Table A2.
[c]Table 10

Analytical composition						
NITROGEN CONTENT (g/kg dry matter)	19.69	34.19	33.29	34.57	34.91	27.28
AME(N) CONTENT (MJ/kg dry matter)	14.54	14.06	12.22[d]	13.91	14.80	14.54

[d]calculated per kg fresh matter.

Amino acid content (g/kg dry matter)						
THREONINE	6.70	7.56	6.21	7.83	7.49	7.06
GLYCINE	10.17	11.17	8.34	16.21	11.88	10.35
CYSTINE	0.98	2.72	2.49	2.36	2.13	1.52
METHIONINE	7.38	7.43	6.32	7.52	7.61	8.74
LEUCINE	13.91	15.85	13.22	15.45	22.40	14.49
ISOLEUCINE	7.64	8.95	8.72	8.22	7.57	7.86
VALINE	9.00	9.93	6.91	9.50	8.61	9.67
TYROSINE	2.70	8.40	6.54	7.75	7.59	4.09
PHENYLALANINE	3.06	9.90	7.46	9.22	9.34	4.16
TRYPTOPHAN	1.90	2.75	2.36	2.40	1.26	2.38
LYSINE	11.75	12.69	11.44	12.36	11.70	11.97
HISTIDINE	5.13	5.46	4.11	5.06	4.78	5.31
ARGININE	10.74	14.09	12.04	14.42	11.20	11.36

Table 10. Composition of amino acid mixtures incorporated into control diets (g/kg diet)

AMINO ACID	MIX C.	MIX D.	MIX E.
L-THREONINE	4.20	1.00	1.80
GLYCINE	1.90		
DL-METHIONINE	5.80	3.50	4.00
L-LEUCINE	6.90		
L-ISOLEUCINE	5.20	2.50	1.00
L-VALINE	5.70		1.00
L-TRYPTOPHAN	1.30	0.50	
L-LYSINE.HCl	8.60	5.00	6.90
L-HISTIDINE.HCl	3.20		1.40
L-ARGININE.HCl	5.60	2.40	2.40
	48.40	14.90	18.5

Table 11. Mixture of indispensable amino acids added to control diet of experiment 6 in order to create an amino acid imbalance with respect to tyrosine and phenylalanine

AMINO ACID	AMOUNT ADDED (g/kg diet)
L-THREONINE	7.40
L-GLYCINE	14.00
DL-METHIONINE	9.20
L-LEUCINE	14.70
L-ISOLEUCINE	8.50
L-VALINE	9.80
L-TRYPTOPHAN	2.10
L-LYSINE.HCl	13.80
L-HISTIDINE.HCl	6.00
L-ARGININE.HCl	12.40
	97.90

sources of zinc. All eight diets were analysed for zinc content by the Central Analytical Laboratory of The East of Scotland College of Agriculture. The experiment was arranged in a randomised block design and birds were killed after fourteen days of feeding the diets.

g. Experiment 7. Effect of ephedrine on a dietary tyrosine+phenylalanine imbalance

Aims

It was determined to investigate the effect of ephedrine, a thermogenic compound known to increase the release of NE from storage sites (Kruk and Pycock, 1979), on the amino acid imbalance with respect to the catecholamine precursors as previously discussed.

Procedure

Zinc-adequate diets were composed as before but phenylalanine supplementation when required was made only at the level of 10g/kg. These diets were fed with and without the incorporation of L-ephedrine hydrochloride at a concentration of 1g/kg. Incorporation of drugs into the diet ensured continual administration and removed the requirement for other methods of dosing the chicks which would be both time-consuming and stressful for the birds (Dulloo and Miller, 1984). Composition of the control diet and the imbalancing amino acid mixture added was as for the previous investigation (Tables 9, 10 and 11) except that the minerals+choline mixture employed was zinc-adequate throughout this experi ment. The experiment was again of a randomised block design and all chicks were killed at the end of the experimental period.

h. Experiment 8. The effects of increased incorporation of balanced and imbalanced proteins into the diet

Aims

The presentation of a high-protein diet to rats initially results in a fall in food intake and growth, followed by a gradual adaptation and restoration of both food intake and weight gain (Peng, Meliza, Vavich and Kemmerer, 1974). It was decided therefore to investigate the effects of a high-protein diet on the performance and brain neurotransmitter concentrations of chicks, and also to compare the effects of feeding high concentrations of protein as supplied by the imbalanced gelatin with those of feeding similar concentrations of protein supplied by the balanced casein.

Procedure

Chicks were thus fed diets of varying protein content, formulated by addition of either gelatin or casein to a control diet, the composition of which is shown in Table 9. In an attempt to avoid a high level of incorporation of gelatin causing stickiness of diets, that employed here was of the lowest 'bloom' value available, that is, formed the least strong gels. Additions as indicated in Table 12 were made at the expense of an equal quantity of glucose, with cellulose being incorporated at a concentration of 20g/kg in order to minimise variation in calculated dietary AME. Pairs of diets were in this way formulated to incorporate gelatin or casein at concentrations intended to raise the dietary nitrogen content by 16g/kg, 29g/kg or 48g/kg. The experiment was conducted in a randomised block arrangement, all chicks being sacrificed after fourteen days of feeding the diets, and heads collected as stated previously.

Table 12. The casein, gelatin, and cellulose contents of diets containing high concentrations of balanced and imbalanced proteins

Ingredient	DIET II	III	IV	V	VI	VII
Cellulose	20.00	20.00	20.00	20.00	20.00	20.00
Casein	107.40	194.60	322.20			
Gelatin				101.90	184.70	305.80
Calculated nitrogen content (g/kg)	49.28	62.28	81.28	49.28	62.28	81.28

j. Experiment 9. The leucine-valine antagonism

Aims

In the chick, the depressed growth and food intake caused by incorporation of too high a concentration of leucine in the diet has been shown to be accompanied by a fall in the plasma concentrations of isoleucine and valine (Smith and Austic, 1978). Valine supplementation has the greater effect in alleviating adverse symptoms, although addition of the other branched-chain amino acid, isoleucine, is also of benefit (D'Mello and Lewis, 1970b). In the rat, an excess of dietary leucine was shown to deplete brain levels of 5HT and DA, while addition of supplementary isoleucine opposed these effects (Yuwiler and Geller, 1965; Geller and Yuwiler, 1967; Krishnaswamy and Raghuram, 1972). It was therefore decided to determine what effect the phenomenon of leucine-valine antagonism might have on the brain neurotransmitter concentrations of the chick.

Procedure

To a control diet constituted so as to be marginally limiting in valine for growth was added valine at a concentration of 1.2g/kg, leucine at a concentration of 40g/kg or an identical amount of leucine in combination with valine at 7g/kg. Composition of the control diet is shown in table 9. All amino acid additions were again made at the expense of an isonitrogenous quantity of glutamic acid. The experimental arrangement constituted two 4x4 Latin squares, chicks of one being killed and heads taken at two points during the feeding period, and those of the second being taken as feeding was terminated.

k. Experiment 10. Effects of supplementary tryptophan and phenylalanine on the response of the chick to an increased dietary content of the branched-chain amino acids

Aims

While the alleviatory effect of supplemental valine on a dietary excess of leucine is well documented, the possible effects of raising the levels of incorporation of all three branched-chain amino acids (BCAA) leucine, isoleucine and valine while maintaining their relative proportions are not. Maintaining the same ratio of the BCAA to each other in terms of proportions of their estimated requirements should prevent any effects due to excessive levels of one of these alone. However a high concentration of a mixture of the three may itself have an effect on chick growth and food intake. As the BCAA are reported to be in competition with tryptophan, tyrosine and phenylalanine for transport across the blood-brain barrier (see Chapter 1), an indirect effect on chick brain neurotransmitter concentrations might perhaps be expected due to changes in uptake of their precursors into the brain. Benton, Harper, Spivey and Elvehjem (1956) did in fact report interactions between phenylalanine, isoleucine and valine which affected the growth and food intake of the weanling rat.

Procedure

Thus to a control diet providing adequate amounts of all indispensable amino acids (Table 9) a mixture of leucine, isoleucine and valine was added in increasing amounts at the expense of equal quantities of glucose. This mixture supplied the three amino acids at the same percentage of their respective requirements. The effect of supplementary phenylalanine at a level of 8g/kg, tryptophan at a level of 4g/kg or the combined effect of both was also tested by making

additions of these at the expense of glucose to certain of the BCAA-supplemented diets. Additions in this investigation were made at the expense of glucose in order to avoid the incorporation of large quantities of glutamic acid being necessary. The experiment was of a randomised block design, all chicks being killed and heads collected at the end of the feeding period.

1. Experiment 11. Excessive dietary content of the branched-chain amino acids and increased supplementation with phenylalanine and tryptophan

Aims

Data obtained from the above experiment indicated that additional dietary tryptophan and phenylalanine might indeed be of some benefit in alleviating the adverse effects of excessive dietary concentrations of the BCAA. A study of the effects of supplementation of the diet with increased concentrations of these two amino acids was therefore made.

Procedure

To a control diet identical to that employed in the previous experiment (Table 9) was added the mixture of BCAA which had been found then to cause the largest depression in chick growth and food intake. The effect of additional phenylalanine at a concentration of 12g/kg either alone or in combination with tryptophan at a concentration of 8g/kg was tested. The four diets thus formulated were fed *ad. lib.*, the experiment being arranged in two 4x4 Latin squares. Feeding was continued over three weeks, two chicks from each cage of one square being killed and heads frozen at two points during this period and those of the remaining square being killed and heads taken on the final day of the experimental period. It was intended that this procedure should provide information as to the validity of a suggestion that the blood-brain barrier of the

chick might not in fact be complete by day seven of life when feeding experiments were begun but continued to develop during the first month (Levi and Morisi, 1971, Purdy and Bondy, 1976). By extending the feeding period and sampling at intervals, the likelihood of this could perhaps be elucidated.

m. Experiment 12.The effect of additional tryptophan on chicks fed high concentrations of dietary phenylalanine

Aims

A possible antagonism of tryptophan by excessive dietary phenylalanine in the chick was reported by Elkin and Rogler (1983). As the former amino acid is the precursor of 5HT and the latter a precursor of NE and DA, this report was considered of particular interest. It was determined to reproduce this effect and investigate the possibility that changes in the concentrations of these neurotransmitters in the brains of chicks accompanied alterations in the dietary concentrations of tryptophan and phenylalanine.

Procedure

A control diet was formulated in which tryptophan was limiting for growth (Table 9) and was fed alone and with a supplement of tryptophan of 1g/kg. The effect of two levels of supplementary tryptophan on two levels of excess phenylalanine was investigated, tryptophan additions of 2g/kg and 4g/kg being made to diets containing supplements of phenylalanine of 20g/kg and 40g/kg. All amino acid additions were made at the expense of an isonitrogenous quantity of glutamic acid. The experiment was of a randomised block design.

n. Experiment 13. The effect of additional phenylalanine on chicks fed high concentrations of dietary tryptophan.

Aims

The existence of a mutual antagonism in which supplementary phenylalanine might alleviate the adverse effects of feeding excessive dietary tryptophan was next investigated.

Procedure

A diet was formulated so as to be limiting in phenylalanine. This required the incorporation of a small additional supplement of potassium and certain vitamins above those amounts incorporated in the mineral and vitamin mixtures employed. The composition of this diet is shown in Table 9. A second diet incorporated a phenylalanine supplement of 8g/kg. To the phenylalanine-limiting diet was added excess tryptophan at a level of 3g/kg, alone and with phenylalanine supplements of 8, 12 and 20g/kg. All amino acid additions were made at the expense of an isonitrogenous quantity of glutamic acid. The experiment was of a randomised block arrangement, chicks being killed and heads collected on the final day of the experiment.

2.4 Statistical analysis of experimental data

Cage means of all data regarding weight gain, food intake or brain concentrations of particular neurotransmitters in chicks fed different diets were analysed statistically by the analysis of variance appropriate to the design of the experiment. Tests of significance between mean values of responses to different treatments were made by Student's t-test (Snedecor and Cochran, 1963), differences being taken to be significant at $P<0.05$, $P<0.01$ and $P<0.001$. This test was also made to determine the effect of the length of the feeding period and hence chick age on brain concentrations of the neurotransmitters and metabolites

where appropriate. Correlation analyses of neurotransmitter concentrations in the brain with the total intake of food, protein, energy or the appropriate amino acid precursor were also performed (Snedecor and Cochran, 1963), by means of a programme designed for a Commodore 'Pet' microcomputer.

It should be noted that the growth and food intake data plotted throughout does not incorporate indications of the limits within which points lie with a probability of 0.95. Such 'confidence limits' have been omitted for the sake of graphical clarity, but all significant differences between the growth and food intake responses of chicks fed the different diets of a particular investigation have been referred to in the text.

Examples of the analyses of data from experiments of Latin square and randomised block design are shown in Tables A4 and A5 respectively of the appendix.

CHAPTER THREE

RESULTS

3.1. Chick mortality during feeding experiments

In most of the experiments undertaken very few deaths occurred and the causes of these were were not attributable to the feeding regime itself. In the case of chicks of experiment 8 however, it was suggested that the nine deaths which occurred were a result of the physical nature of the diets presented. These, due to their high content of purified protein and crystalline amino acids, were of a finely divided, particulate form which was liable to become impacted within the beak, so causing infection and subsequent death to the chick. Similar diets had been presented in experiment 7 but only one death occurred in this case.

3.2. Experiment 1. Feeding of the starter diet

Chicks fed starter diet over the period indicated showed very good growth and food intake (Figs. 12 and 13). Table 13 shows the brain concentrations of the various measured neurotransmitters and metabolites. Those of E and HVA were very low and as the ratios of responses measured at electrode potentials of 0.6V and 0.72V indicated that the purity of these eluted peaks was in doubt, both these sets of observations have been omitted.

For chicks fed the starter diet for fourteen days beyond the usual seven-day period, the only significant difference observed between those killed at different times was in the brain concentration of 5HT. This was lower at 14.00h than at 16.00h ($P<0.05$) but neither of these concentrations was found to be significantly different from that measured at 10.00h. No significant difference in brain concentrations at 10.00h and 14.00h for any of the measured compounds was found after a further seven days.

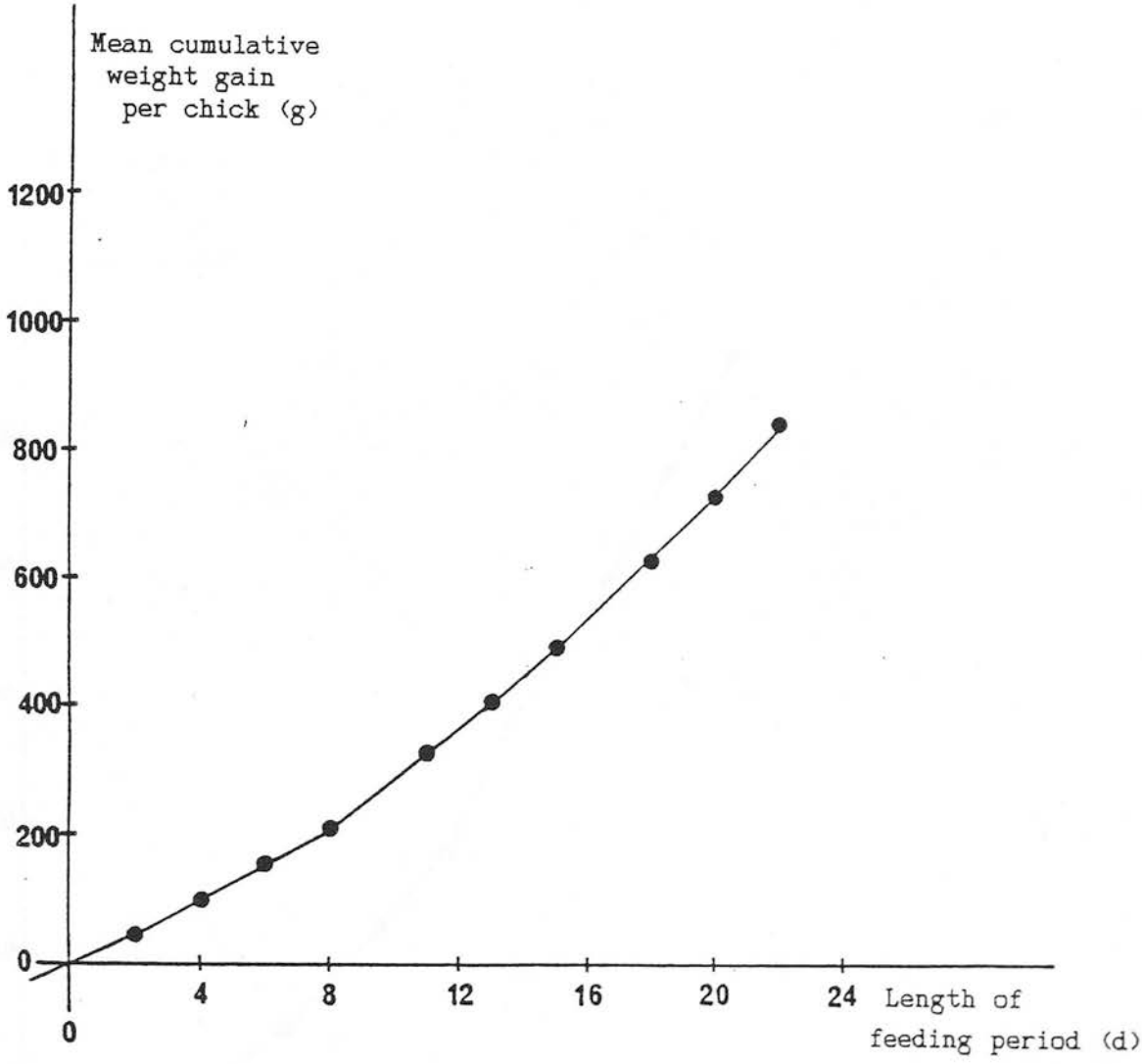


Fig. 12. Weight gain of chicks receiving starter diet.

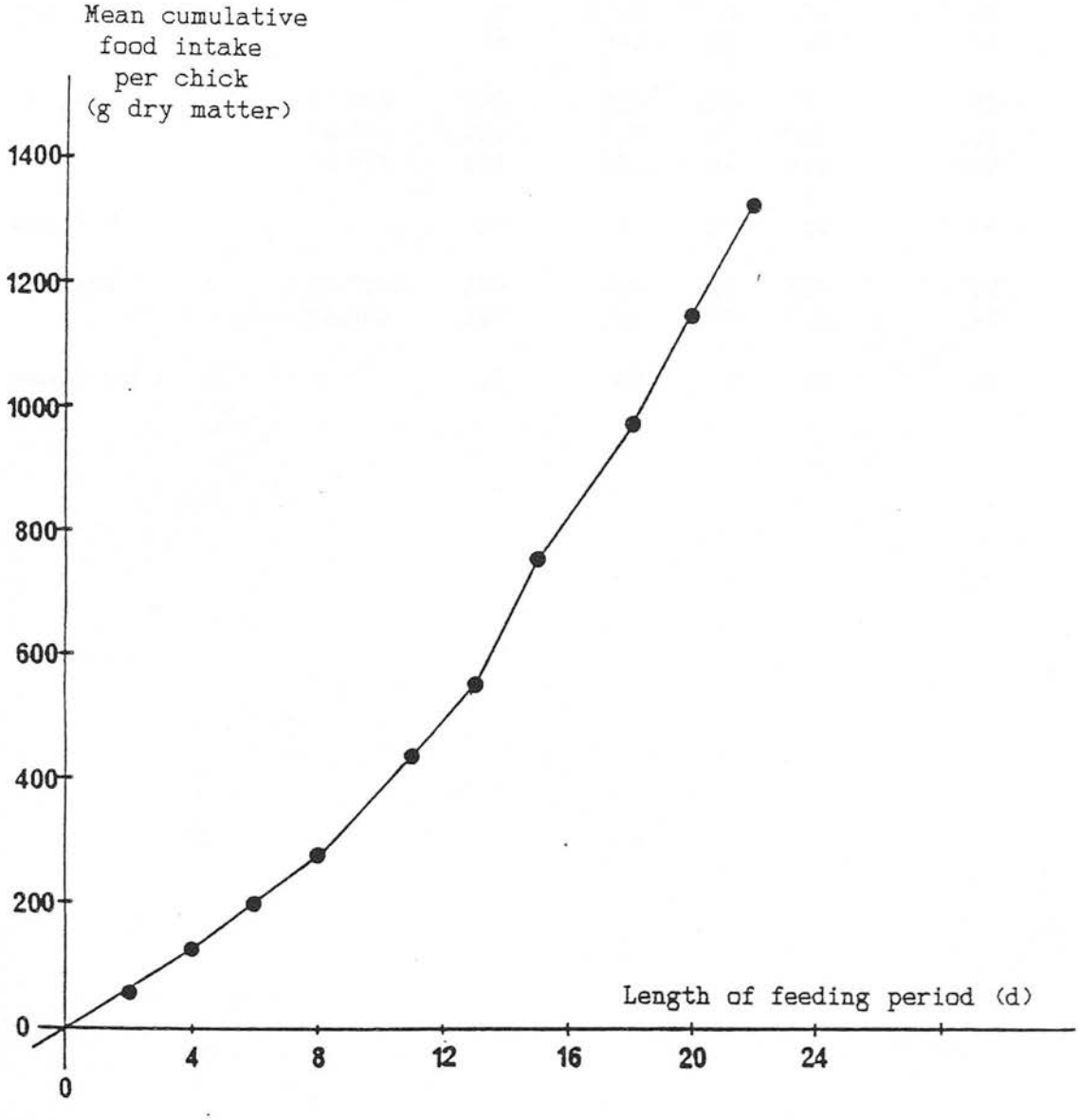


Fig. 13. Food intake of chicks receiving starter diet.

Table 13. Effect of age and time of killing on brain concentrations of neurotransmitters and metabolites in chicks fed starter diet

Length of feeding period (Beyond 7-day norm)	Time	Mean concentration of compound (ng/g fresh wt. brain tissue) NE	DA	DOPAC	5HT	5HIAA
0 days	12.00h	120	84	61	320	121
sem		10	8	6	31	6
d.f.		43	42	33	43	43
14 Days	10.00h	250	262	120	732	95
	14.00h	221	217	93	543	105
	16.00h	304	333	64	918	125
sem(5 d.f.)		26	41	20	64	16
21 Days	10.00h	286	339	66	824	110
	14.00h	247	265	75	713	90
sem(6 d.f.)		27	45	6	98	16

The concentrations of the measured neurotransmitters and metabolites in the brain of the chick showed some variation with chick age and the length of the feeding period. The mean brain concentrations of NE, DA and 5HT of birds fed the starter diet for only seven days were significantly lower than those of chicks which had received this diet for an additional fourteen or twenty-one days ($P<0.001$). Chicks fed the starter diet for only the initial period of seven days also had a lower brain concentration of DOPAC than those receiving the same diet for a further fourteen days ($P<0.05$).

3.3. Experiment 2. The lysine-arginine antagonism

a. Dietary nitrogen content

As shown in Table 14., there was little difference apparent in the nitrogen contents of the four experimental diets employed in this investigation.

b. Growth and food intake (Figs. 14 and 15.)

Chicks fed the diet incorporating the arginine supplement of 3.5g/kg had gained significantly more weight than those receiving the control diet after ten ($P<0.05$), twelve ($P<0.01$) and fourteen ($P<0.05$) days of feeding. Food intake was unchanged throughout however. By the second day of feeding, birds fed the diet containing excess lysine had a lower weight gain ($P<0.01$) and food intake ($P<0.05$) than those fed the control diet or that supplemented with arginine alone, the depression in growth being highly significant thereafter ($P<0.001$). The accompanying reduction in food intake increased in significance on the sixth day of feeding ($P<0.01$), and was subsequently highly significant ($P<0.001$).

Improved growth over that of chicks fed the supplement of excess lysine alone was immediately shown by birds fed a concomitant supplement of arginine ($P<0.05$). The difference in weight gain of chicks

Table 14. Determined nitrogen content of diets of experiments indicated (g/kg dry matter)

Experiment 2.		
I	Control diet marginally deficient in arginine	34.62
II	Control+arginine (3.5g/kg)	35.22
III	Control+excess lysine (15g/kg)	35.50
IV	Control+lysine (15g/kg)+arginine (12g/kg)	34.81
Experiment 3.		
I	Control diet	35.82
II	Control+methionine (2g/kg)	35.98
III	Control+methionine+glycine (1g/kg)	37.37
IV	Control+methionine+glycine+arginine(1g/kg)	36.02
Experiment 4.		
I	Low-tryptophan control diet	33.03
II	Control+L-tryptophan (1.5g/kg)	38.16
III	Control+indispensable amino acid mixture lacking tryptophan	35.53
IV	Control+indispensable amino acid mixture +tryptophan (1.5g/kg)	36.44
Experiment 9.		
I	Control diet having low valine content	33.29
II	Control+valine (1.2g/kg diet)	34.67
III	Control+leucine (40g/kg diet)	34.84
IV	Control+leucine+valine (7g/kg)	34.56

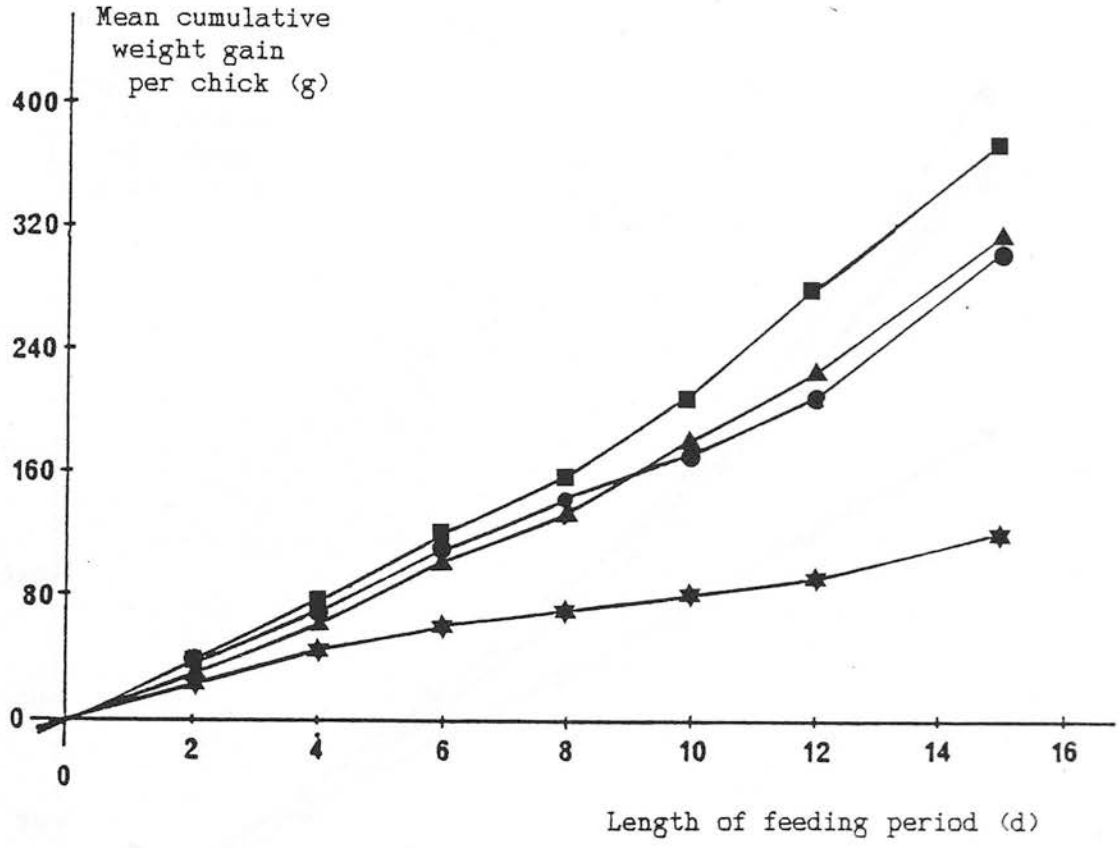


Fig. 14. Weight gain of chicks fed diets containing different amounts of lysine and arginine

- ● I Control diet marginally deficient in arginine
- ■ II Control+arginine (3.5g/kg)
- ★ III Control+excess lysine (15g/kg)
- ▲ IV Control+lysine (15g/kg)+arginine (12g/kg)

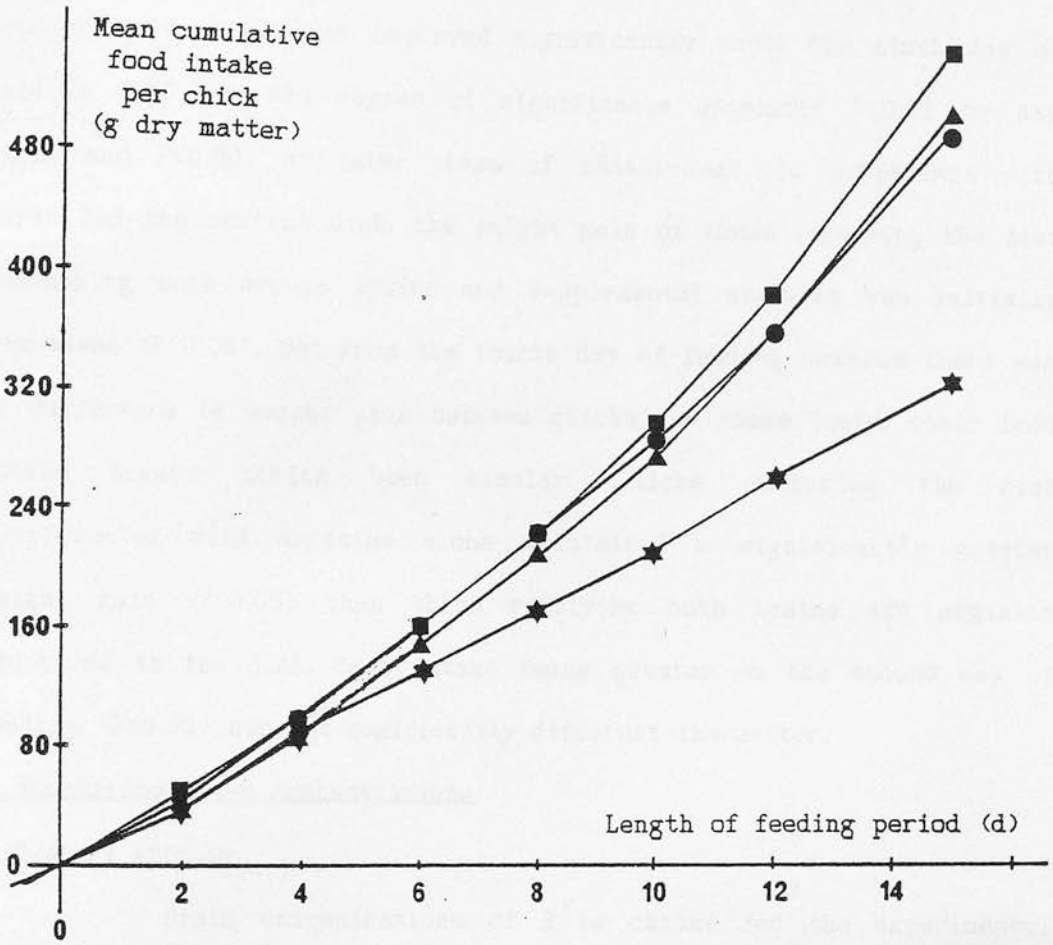


Fig. 15. Food intake of chicks fed diets containing different amounts of lysine and arginine

- ● I Control diet marginally deficient in arginine
- ■ II Control+arginine (3.5g/kg)
- ★ III Control+excess lysine (15g/kg)
- ▲ IV Control+lysine (15g/kg)+arginine (12g/kg)

fed these two diets had achieved greater significance by the fourth day of feeding ($P<0.01$) and was highly significant thereafter ($P<0.001$). Food intake however, was not improved significantly until the sixth day of feeding ($P<0.05$); the degree of significance attaining $P<0.01$ by day eight and $P<0.001$ at later times of measurement. In comparison with birds fed the control diet, the weight gain of those receiving the diet containing both excess lysine and supplemental arginine was initially depressed ($P<0.05$), but from the fourth day of feeding onwards there was no difference in weight gain between chicks fed these diets, their food intake always having been similar. Chicks consuming the diet supplemented with arginine alone maintained a significantly greater weight gain ($P<0.05$) than those receiving both lysine and arginine additions to the diet, food intake being greater on the second day of feeding ($P<0.05$) but not sigificantly different thereafter.

c. Neurotransmitter concentrations

i. Dietary effects

Brain concentrations of E in chicks fed the experimental diets were never sufficiently high to be measureable, while it was not possible to consistently determine HVA concentrations in chicks fed the diets for four days and these values have been omitted. As shown in Table 15., the brain concentrations of NE, DA and 5HIAA in chicks consuming the diet containing both excess lysine and additional arginine were, after four days of feeding, significantly higher than in those receiving that containing excess lysine alone ($P<0.05$), although none of these values was found to be significantly different from the corresponding measurements in chicks fed the other two diets. No significant differences in the brain concentrations of DOPAC or 5HT in chicks fed the different diets were seen at this point.

Table 15. The effects of lysine-arginine antagonism on chick brain concentrations of selected neurotransmitters and metabolites

Diet fed for period indicated	Mean concentration of compound (ng/g fresh wt. brain tissue) NE	DA	DOPAC	HVA	5HT	5HIAA
4 Days						
Diet I	81	104	29	n.d.	499	179
Diet II	123	155	30	n.d.	602	177
Diet III	42	41	35	n.d.	674	125
Diet IV	177	197	28	n.d.	584	188
sem	25	34	4		118	18
d.f.	5	6	6		6	6
8 Days						
Diet I	148	177	30	119	710	179
Diet II	177	218	40	124	838	157
Diet III	167	262	43	154	680	214
Diet IV	169	190	30	105	763	148
sem	14	72	11	30	44	28
d.f.	6	6	5	5	6	6
15 Days						
Diet I	236	198	43	117	850	166
Diet II	282	279*	41	126	764	151
Diet III	227	269	62	223***	731	152
Diet IV	220	221	46	368***	740	98*
sem	47	23	8	10	50	14
d.f.	6	6	5	5	6	6

Values significantly different from control values determined on that day, *P<0.05, **P<0.01, ***P<0.001
n.d.=not determined

I Control diet marginally deficient in arginine
II Control+arginine (3.5g/kg)
III Control+excess lysine (15g/kg)
IV Control+lysine (15g/kg) + arginine (12g/kg)

On the eighth day of feeding, chicks receiving the diet incorporating excess lysine alone had a significantly lower brain concentration of 5HT than those fed that supplemented only with arginine ($P<0.05$). No other significant differences were observed in the brain concentrations of the measured neurotransmitters and metabolites for birds fed the various diets.

Chicks receiving the control diet+arginine showed, by the final day of the experiment, an increase in brain DA concentrations relative only to those fed the control diet alone ($P<0.05$). The brain 5HIAA concentration of chicks fed a dietary excess of lysine together with supplemental arginine was significantly below that of all other chicks ($P<0.05$). No differences in brain 5HT content between the groups of birds were observable. Chicks consuming excess lysine alone showed greater brain concentrations of HVA than those fed the control diet ($P<0.001$) or that having a small amount of arginine incorporated ($P<0.01$). However, birds fed the diet supplemented with both excess lysine and additional arginine had higher brain concentrations of HVA than those fed any of the other diets ($P<0.001$).

ii. Effects of age and length of feeding period

Certain changes in the brain concentrations of some of the measured compounds with age or duration of the feeding period were apparent. The brain concentration of NE in birds which had been fed the diet incorporating excess lysine alone for a period of eight days was greater than that of those consuming the same diet for only four days ($P<0.01$). Chicks fed the control diet for fifteen days had significantly higher brain concentrations of DOPAC ($P<0.05$) and HVA ($P<0.001$) than those which had consumed it for eight days. After fifteen days of receiving the diets, birds fed the control diet had higher brain

concentrations of NE, DOPAC and 5HT than those receiving the diet for four days only ($P<0.05$). Chicks consuming the diet supplemented with arginine alone also had a significantly greater brain NE concentration after fifteen days of feeding than after only four days of the experimental period ($P<0.05$). Those fed the diet incorporating excess lysine alone had higher brain concentrations of both NE ($P<0.001$) and DA ($P<0.01$) at the end of the feeding period than after only four days of feeding.

iii. Neurotransmitter concentrations and food intake

After eight days of the feeding period, chick brain concentrations of 5HT were positively correlated with their intake of food as a whole or tryptophan in particular ($r=0.521$, $P<0.05$), but were not significantly correlated with their actual protein intake. No such correlations were observed at any other time. Chick brain concentrations of NE or DA were at no time correlated with the total intake of food, combined intake of tyrosine+phenylalanine or protein intake .

3.4. Experiment 3. The effect of supplementary glycine and arginine on methionine toxicity

a. Dietary nitrogen content

Some difference in the determined nitrogen contents of the diets fed during this investigation was apparent (Table 14), the greatest difference being one of 1.55g/kg between the control diet and that to which both excess methionine and additional glycine had been added.

b. Growth and food intake (Figs. 16 and 17.)

The control diet employed here supported good growth and food intake. Addition of excess methionine had a very detrimental effect, a significant depression in both weight gain ($P<0.001$) and food intake

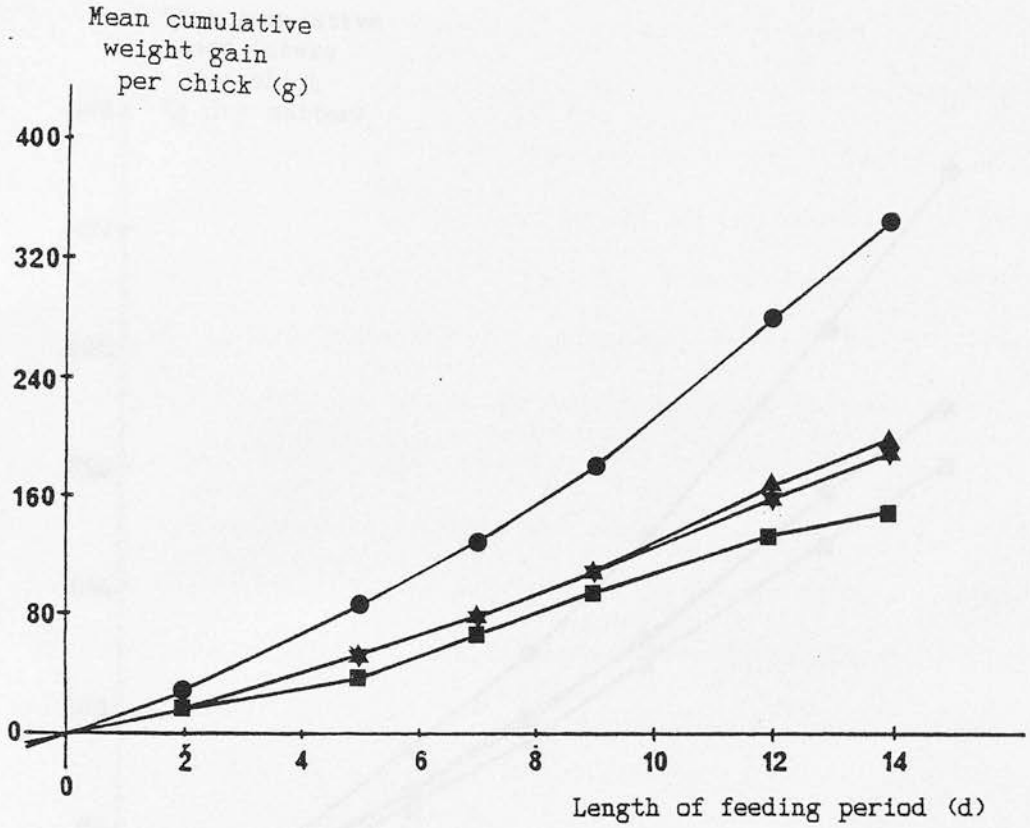


Fig. 16. Weight gain of chicks fed methionine alone and with supplemental glycine and arginine

- ● I Control diet adequate in all indispensable amino acids
- ■ II Control+methionine (2g/kg)
- ★ III Control+methionine+glycine (1g/kg)
- ▲ IV Control+methionine+glycine+arginine (1g/kg)

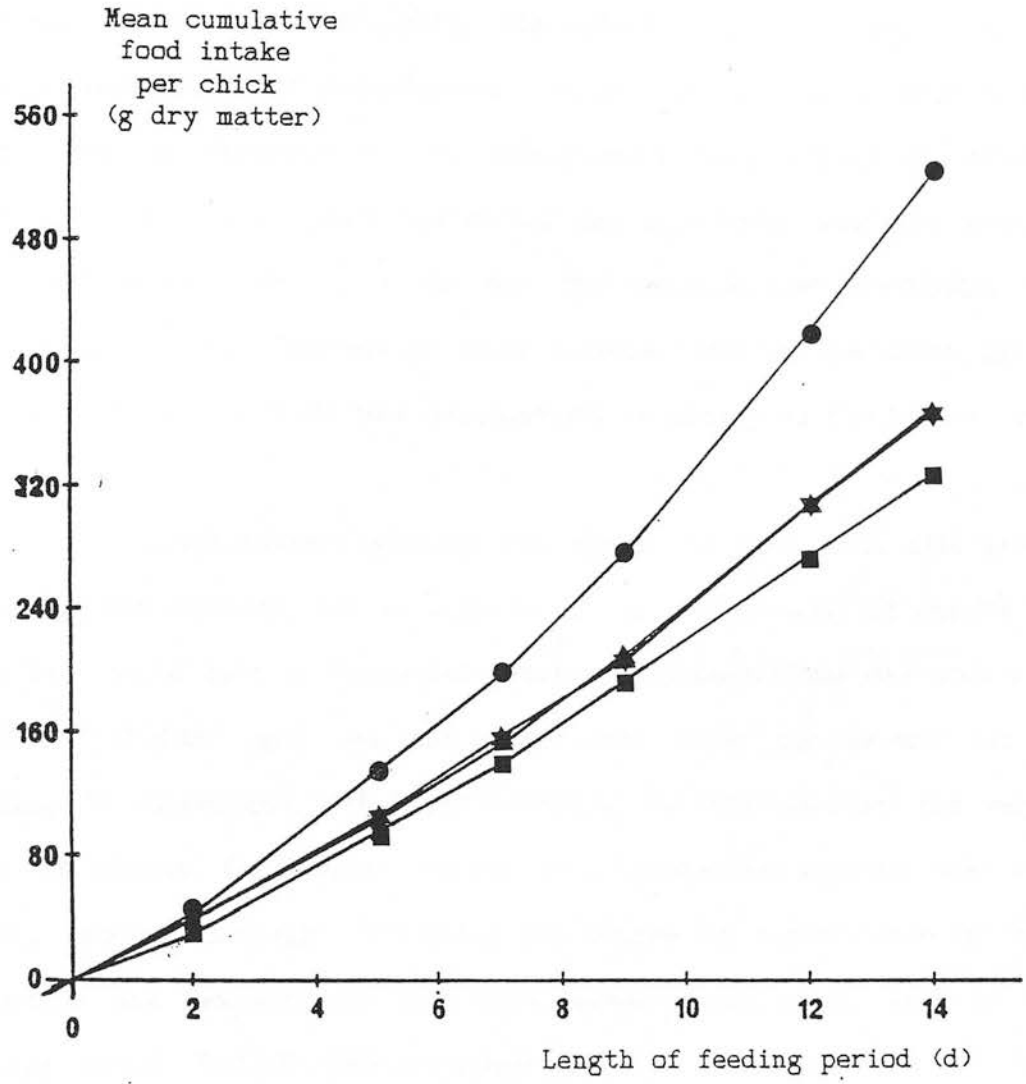


Fig. 17. Food intake of chicks fed methionine alone and with supplemental glycine and arginine

- ● I Control diet adequate in all indispensable amino acids
- ■ II Control+methionine (2g/kg)
- ★ III Control+methionine+glycine (1g/kg)
- ▲ IV Control+methionine+glycine+arginine (1g/kg)

($P<0.01$) below those of chicks fed the control diet being observed after only two days of food consumption. The reduction in weight gain attained only a lower level of significance ($P<0.01$) on the fifth, seventh and ninth days of feeding but was subsequently very highly significant ($P<0.001$). Food intake after the second day of feeding was very greatly depressed below that of birds fed the control diet ($P<0.001$), the significance of this depression being lessened only on the ninth day of food consumption ($P<0.01$) and subsequently returning to the higher level ($P<0.001$).

Supplementary glycine did appear to have some alleviatory effect on the toxicity, but an improvement in weight gain of chicks fed this diet above that of those fed excess methionine alone was seen only initially ($P<0.05$) and was not significant after the second day of feeding. In comparison with birds receiving the control diet, the weight gain of chicks fed excess methionine+supplemental glycine was very highly reduced initially ($P<0.001$). The degree of significance of this reduction was lessened on the fifth, seventh and ninth days of the feeding period ($P<0.05$), and increased somewhat thereafter ($P<0.01$). Only on the fifth and seventh days of the feeding period did chicks fed excess methionine with supplemental glycine show an increase in food intake above that of those fed excess methionine alone ($p<0.05$). Their food intake was below that of those consuming the control diet by the second day of feeding ($P<0.05$) and was subsequently highly reduced ($P<0.001$), the significance of this reduction being lessened on and after the ninth day of the investigation ($P<0.01$).

Incorporation of arginine in addition to glycine and excess methionine appeared to have no additional effect on chick growth and food intake, these measurements being not significantly different from

those of birds fed only methionine+glycine. Only on the second day of the feeding period was the weight gain of chicks fed excess methionine+glycine+arginine significantly greater than that of birds fed excess methionine alone ($P<0.05$), while food intake showed an improvement only on the seventh day ($P<0.05$). Chicks receiving the control diet consistently had a greater weight gain and food intake than those fed methionine+glycine+arginine, the significance of these differences being identical to those between birds fed the control diet and those fed methionine+glycine without arginine supplementation.

c. Neurotransmitter concentrations

i. Dietary effects

The response ratio calculated during the determination of E indicated that this eluted peak was likely to contain contaminants and determinations of this compound were not therefore made. As shown in Table 16, no significant differences between the brain concentrations of NE or 5HIAA in chicks fed the various diets were observable at any time. After 5 days of feeding, only DOPAC and 5HT showed any variation between the chick groups, that group receiving excess dietary methionine with glycine having a brain content of DOPAC which was greatly increased above that of all other groups ($P<0.001$). Chicks receiving supplements of both glycine and arginine also had a higher brain concentration of DOPAC than those fed the control diet ($P<0.05$). The brain 5HT content of chicks fed excess methionine with glycine was below that of those consuming the control diet or that containing only additional methionine ($P<0.01$), or that incorporating excess methionine and supplementary glycine and arginine ($P<0.05$). Chicks fed this latter diet also had lower brain concentrations of 5HT than those fed the control diet ($P<0.05$).

Table 16. Brain concentrations of neurotransmitters and metabolites in chicks fed excess methionine alone or with supplemental glycine and arginine

Diet fed for period indicated	Mean concentrations of compound (ng/g fresh wt. brain tissue) NE	DA	DOPAC	HVA	5HT	5HIAA
5 Days						
Diet I	215	208	35	125	814	115
Diet II	265	204	40	175	779	111
Diet III	264	230	115***	184	604**	106
Diet IV	221	201	65*	160	715*	145
sem (6 d.f.)	46	38	9	35	26	20
9 Days						
Diet I	220	201	39	113	710	110
Diet II	203	129	68	146	585	119
Diet III	222	170	99	179*	563	133
Diet IV	199	127	49	175*	606	133
sem (6 d.f.)	18	35	18	16	58	13
14 Days						
Diet I	205	137	56	110	636	106
Diet II	212	76	39	126	748	103
Diet III	180	165	82	174**	512	127
Diet IV	190	103	100	154*	511	116
sem (6 d.f.)	24	39	15	11	47	9

Values significantly different from control values, *P<0.05, **P<0.01, ***P<0.001

I Control diet adequate in all indispensable amino acids
II Control+methionine (2g/kg)
III Control+methionine+glycine (1g/kg)
IV Control+methionine+glycine+arginine (1g/kg)

On the ninth day of feeding the experimental diets, chicks receiving excess methionine and supplementary glycine with or without additional arginine, had higher brain concentrations of HVA than those fed the other two diets ($P<0.05$). No other differences in the brain concentrations of any of the other measured compounds were observable between chicks fed the different diets.

By the final day of the experiment, the brain concentration of DOPAC in chicks fed extra dietary methionine+glycine+arginine was significantly above that of those receiving excess methionine alone ($P<0.05$). The brain DA concentration of the former group of birds was below that of those fed either the latter diet or that containing both excess methionine and additional glycine in the absence of supplemental arginine ($P<0.05$). Brain concentrations of HVA in chicks receiving methionine+glycine were significantly greater than in birds fed either the control diet ($P<0.01$) or that incorporating excess methionine alone ($P<0.05$). Chicks fed additional methionine+glycine+arginine also had a higher brain concentration of HVA than those fed the control diet ($P<0.05$).

The concentration of 5HT in the brains of chicks receiving excess methionine alone was significantly greater than that of birds fed diets supplemented with glycine, with or without additional arginine ($P<0.05$). No significant difference in the brain 5HIAA concentrations of chicks fed the various diets was observed.

ii. Effects of age and length of feeding period

There was no significant effect of the length of the feeding period and hence age of the chick on the brain concentrations of the measured compounds in birds fed any one diet.

iii. Neurotransmitter concentrations and food intake

The brain concentrations of NE, DA and 5HT in chicks receiving the experimental diets were not significantly correlated with their total food intake or the quantity of protein consumed. No correlations of the brain concentration of 5HT with tryptophan intake, or the brain concentrations of NE or DA with the combined intake of tyrosine+phenylalanine were observed.

3.5. Experiment 4. The induction of a dietary tryptophan imbalance

a. Dietary nitrogen content

The nitrogen contents of diets formulated for this investigation (Table 14) showed differences of upto 5g/kg.

b. Growth and food intake (Figs. 18. and 19.)

The control diet supported poor growth and food intake. Administration of tryptophan immediately improved weight gain ($p<0.01$), the difference being very highly significant by the seventh day of feeding ($P<0.001$). Food intake was immediately very significantly improved ($P<0.001$). Addition of the mixture of amino acids lacking tryptophan to the control diet resulted in no significant change in weight gain compared with that supported by the control diet alone and only an initial reduction in food intake ($P<0.05$) which was not significant by the fifth day of feeding. Both the weight gain and food intake of birds fed this diet were depressed below that of those fed the diet supplemented with tryptophan alone, the significance of these reductions being at the highest level ($P<0.001$) except for that of the reduction in weight gain on the fifth day of the feeding period, which attained significance at $P<0.01$.

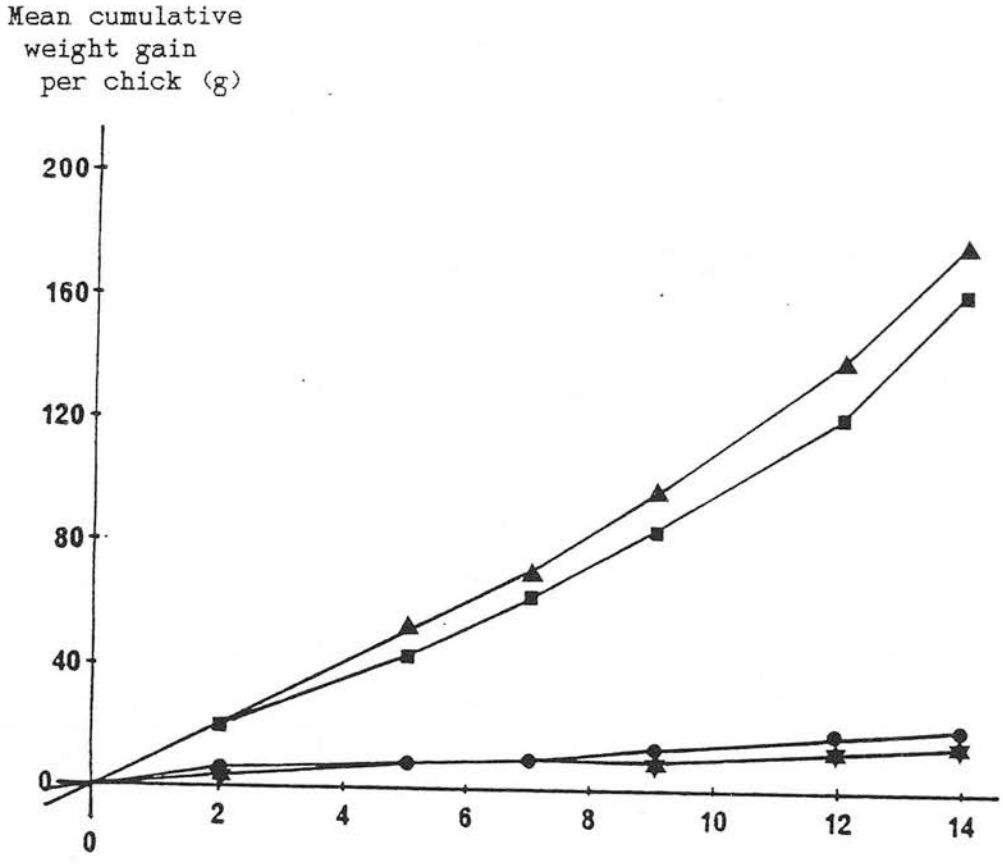


Fig. 18. Weight gain of chicks fed during the study of induction of a dietary tryptophan imbalance

- ●I Low-tryptophan control diet
- ■II Control+L-tryptophan (1.5g/kg diet)
- ★III Control+indispensable amino acid mixture lacking tryptophan
- ▲IV Control+indispensable amino acid mixture+tryptophan (1.5g/kg diet)

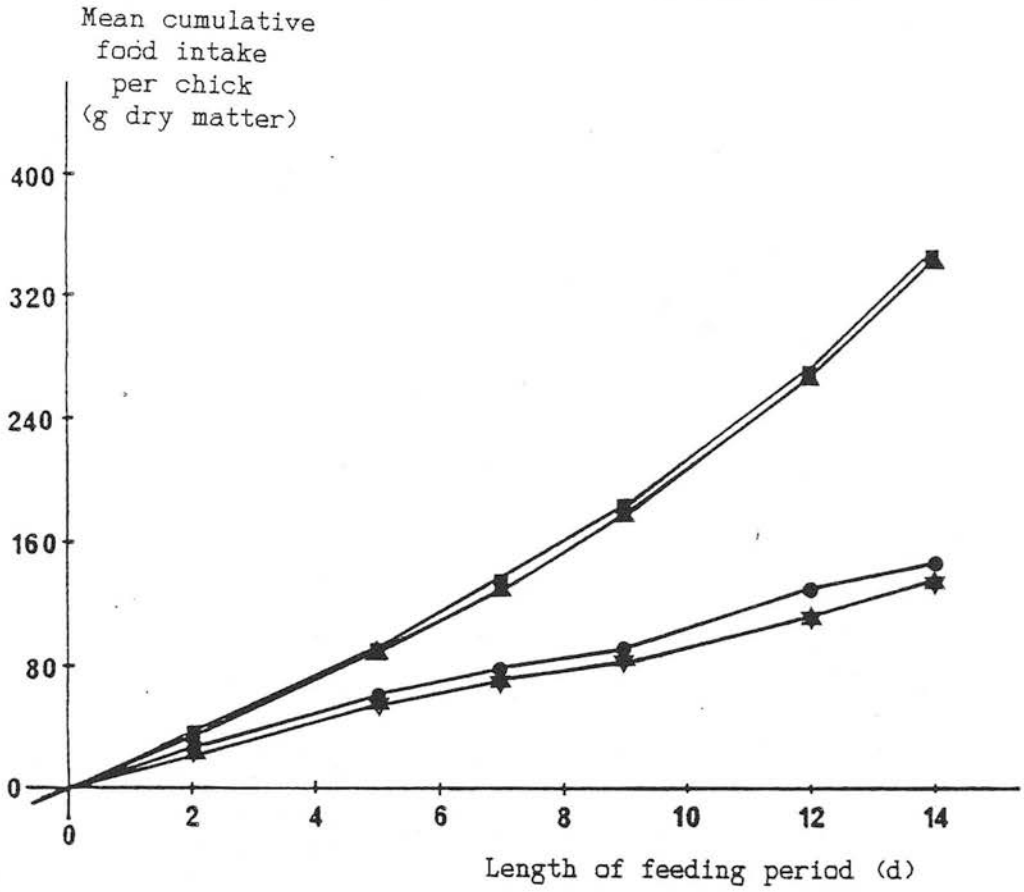


Fig. 19. Food intake of chicks fed during the study of induction of a dietary tryptophan imbalance

- ● I Low-tryptophan control diet
- ■ II Control+L-tryptophan (1.5g/kg diet)
- ✱ III Control+indispensable amino acid mixture lacking tryptophan
- ▲ IV Control+indispensable amino acid mixture+tryptophan (1.5g/kg diet)

Additional tryptophan in combination with the amino acid mixture lacking this amino acid returned growth and food intake to that given by the diet which had been supplemented with tryptophan alone. Weight gain and food intake of chicks fed the former diet were immediately significantly greater than those of birds fed the control diet ($P<0.01$), the improvement being very highly significant thereafter ($P<0.001$). In comparison with chicks fed the diet incorporating only the amino acid mixture lacking tryptophan, both the weight gain and food intake of chicks fed this mixture with supplemental tryptophan were constantly greatly increased ($P<0.001$).

c. Neurotransmitter concentrations

i. Dietary effects

Throughout the experimental period no change in the brain concentrations of NE, E, DOPAC or DA was observed (Table 17). After five days of consuming the diets, significantly higher brain concentrations of HVA were recorded in chicks receiving additional tryptophan alone ($P<0.01$) or with the added amino acid mixture ($P<0.05$) than in those fed the control diet. However, the brain concentrations of HVA in chicks fed all diets but the control were not significantly different from each other. Concentrations of 5HT and 5HIAA in the brains of chicks fed either of the two diets containing additional tryptophan were greater than those of the groups receiving diets which were not tryptophan-supplemented ($P<0.05$ for 5HT and $P<0.001$ for 5HIAA). The brain concentration of 5HIAA was also greater in the group of chicks fed the tryptophan supplement alone than when both tryptophan and the amino acid mixture lacking this amino acid were fed together ($P<0.01$).

Table 17. Chick brain concentrations of neurotransmitters and metabolites during the study of a dietary tryptophan imbalance

Diet fed for period indicated	Mean concentration of compound (ng/g fresh wt. brain tissue) NE	E	DA	DOPAC	HVA	5HT	5HIAA
5 Days							
Diet I	215	19	220	36	73	345	25
Diet II	248	19	184	46	152**	628*	125***
Diet III	245	13	151	29	108	350	28
Diet IV	236	19	240	37	128*	613*	83***
sem (6 d.f.)	43	8	62	11	13	55	6
9 Days							
Diet I	240	19	237	28	84	360	29
Diet II	163	13	250	38	108	688**	112
Diet III	248	17	237	24	108	326	110
Diet IV	178	10	203	22	80	708**	79
sem (6 d.f.)	27	4	25	5	11	55	32
14 Days							
Diet I	248	18	271	33	78	453	39
Diet II	192	16	205	28	118**	796*	118**
Diet III	184	19	215	43	87	300	39
Diet IV	218	17	203	30	124**	548	99*
sem (6 d.f.)	29	4	22	5	6	95	12

Values significantly different from control values *P<0.05, **P<0.01, ***P<0.001

I	Low-tryptophan control diet
II	Control+L-tryptophan (1.5g/kg diet)
III	Control+indispensable amino acid mixture lacking tryptophan
IV	Control+indispensable amino acid mixture+tryptophan (1.5g/kg diet)

By the ninth day of feeding the experimental diets, none of the observed differences in brain concentrations of HVA or 5HIAA for birds consuming the various diets was significant. However, those chicks receiving additional tryptophan still had higher brain concentrations of 5HT than those which did not ($P<0.01$). Incorporation of the tryptophan-lacking amino acid mixture into the diet had no additional effect.

On the fourteenth and final day of the experiment, the brain concentration of HVA in chicks fed the diet containing supplemental tryptophan alone was significantly greater than that of birds consuming either the control diet ($P<0.01$) or that incorporating only the tryptophan-lacking amino acid mixture ($P<0.05$). Chicks fed these latter diets also showed reduced brain concentrations of HVA compared with those fed both this amino acid mixture and additional tryptophan ($P<0.01$).

Concentrations of 5HT in the chick brain were also increased by tryptophan supplementation. When this amino acid was the sole supplement, a significant increase in the brain concentration of 5HT above that of chicks fed either of the diets lacking additional tryptophan was observable ($P<0.05$). However, when fed in combination with the amino acid mixture lacking tryptophan the measured brain concentration of 5HT was not significantly different from values obtained for birds receiving any of the other three diets. Brain concentrations of 5HIAA were elevated in chicks receiving additional tryptophan in the presence ($P<0.05$) or absence ($P<0.01$) of further amino acid supplementation, compared with birds fed the other two diets.

ii. Effects of age and length of feeding period

The brain concentration of HVA in chicks consuming the diet incorporating both the indispensable amino acid mixture and additional

tryptophan was greater after fourteen days of feeding than after nine ($P<0.05$). No other significant differences were observed between the brain concentrations of the measured compounds in chicks fed any one of the diets presented for the different lengths of time.

iii. Neurotransmitter concentrations and food intake

After five days of feeding the diets, the chick brain concentration of 5HT was positively correlated with the level of food intake ($r=0.787$) and the quantity of tryptophan ($r=0.831$) or protein ($r=0.803$) consumed. These correlations were all very highly significant ($P<0.001$). A similar pattern was apparent after nine days of the experimental period, the chick brain concentration of 5HT being positively correlated with food intake ($r=0.773$), tryptophan intake ($r=0.814$) and protein intake ($r=0.776$). These correlations again attained a very high level of significance ($P<0.001$). On the final day of the experimental period, the chick brain concentration of 5HT was again significantly and positively correlated with total food intake ($r=0.624$, $P<0.01$), tryptophan intake ($r=0.597$, $P<0.05$) and the quantity of protein consumed ($r=0.635$, $P<0.01$). At no time was there a significant correlation of the chick brain concentrations of NE or DA with the quantities of food, tyrosine+phenylalanine or protein consumed.

3.6. Experiment 5. Amino acid imbalance and responses to tryptophan supplementation

a. Dietary tryptophan content

It should be noted that the tryptophan-supplemented diet intended to be adequate in this amino acid according to current estimates of the chick's requirement was found to have been formulated erroneously. This diet (IV) supported a much poorer level of growth and

food intake than expected for a tryptophan-adequate diet. While this effect alone would not necessarily be proof of an error in the formulation of the diet, it was also found to be approximately 100g over the expected total weight of 10kg and slightly paler in colour than the others employed in this experiment. For these reasons the altered response of chicks to this diet cannot be taken to be entirely due to the altered tryptophan content. Thus while data regarding the performance and brain neurotransmitter concentrations of chicks fed this diet have been included during the statistical analysis of the experiment as a whole, they are not themselves presented.

b. Variations in dietary nitrogen and AME(N) content

The nitrogen content of the diet formulated by the graded supplementation method to provide tryptophan at 0.48 of its required concentration was apparently greater than that of the other supplemented diets by approximately 6g/kg (Table 18). For the diluted series of diets, nitrogen content fell with increasing dilution, that is, as the tryptophan content of the diet was reduced.

The diluted diet containing tryptophan at 0.63 of its required concentration had a greater determined AME(N) content than diets formulated by the same method to have a greater tryptophan content, and that of the supplemented series providing tryptophan at 0.48 of its required concentration ($P<0.05$). The diet of the diluted series which contained 0.48 of the required tryptophan content similarly had a greater AME(N) than these diets ($P<0.01$). That diluted diet which had been designed to be adequate in its content of tryptophan had a significantly lower AME(N) content than diets of the supplemented series containing 0.63 or a greater proportion of the tryptophan requirement ($P<0.05$).

Table 18. Nitrogen and AME(N) content of diets differing in their amino acid balance and tryptophan content.

	DIET						
	I	II	III	V	VI	VII	VIII
NITROGEN CONTENT (g/kg dry matter)	38.70	33.72	32.70	22.15	30.29	38.16	49.40
AME(N) (MJ/kg dry matter)	13.46	14.02	13.92	14.35**	14.07*	13.48	13.32

sem (21 d.f.)=0.20

Values significantly different from values for diet I, *P<0.05, **P<0.01

I and V, formulated to provide 0.48 of tryptophan requirement of the chick
II and VI, to provide 0.63 of tryptophan requirement
III and VII, to provide 0.78 of tryptophan requirement
VIII, to provide total tryptophan requirement

I-III formulated by the method of graded supplementation
V-VIII formulated by the method of diet dilution

c. Growth and food intake (Figs. 20 and 21.)

Chicks fed the diet of the supplemented series providing 0.48 of the tryptophan requirement initially showed a lower weight gain than those fed the diluted diet supplying the same amount of this amino acid ($P<0.05$). After six days of feeding however, the difference in weight gain of the two groups of birds was no longer significant. The food intake of chicks fed the diet of the supplemented series was initially much less than that of those receiving the diluted diet tenth day of the feeding period the observed difference in food intake was not significant, but subsequently chicks fed the diet of the supplemented series maintained a lower food intake than those fed the diluted diet ($P<0.05$)

The weight gain of chicks fed the supplemented diet containing 0.63 of the required amount of tryptophan was immediately greater than that of those fed the diet of the supplemented series providing 0.48 of the requirement ($P<0.001$). However, this increase in weight gain attained lower levels of significance on the fourth ($P<0.01$) and sixth day ($P<0.05$) of the feeding period, and was not significant on the tenth day. Thereafter, the greater weight gain of chicks fed the higher concentration of tryptophan was significant at the level of $P<0.01$. The food intake of these birds was always greater than that of chicks receiving the diet of the supplemented series containing tryptophan at 0.48 of its required concentration, the difference attaining significance at the level of $P<0.05$ on the sixth, eighth and tenth days of the feeding period and at $P<0.01$ at all other times. In comparison with chicks consuming the diluted diet in which tryptophan was at its lowest concentration, birds receiving the supplemented diet incorporating tryptophan at 0.63 of its requirement showed a

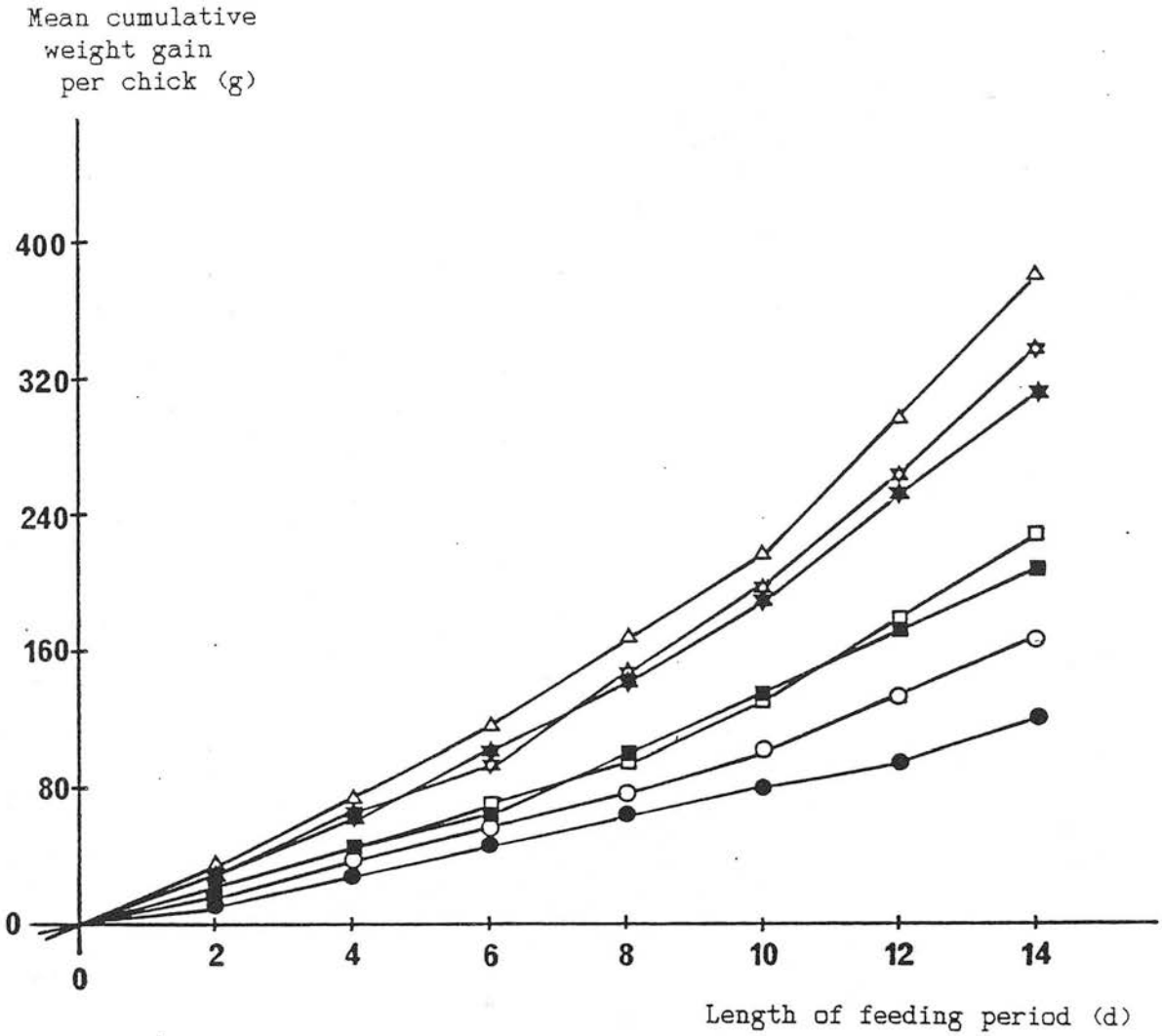


Fig. 20. Weight gain of chicks fed diets differing in their amino acid balance and tryptophan content.

● ○ I and V, formulated to provide 0.48 of tryptophan requirement
■ □ II and VI, to provide 0.63 of tryptophan requirement
★ ✡ III and VII, to provide 0.78 of tryptophan requirement
△ VIII, to provide total tryptophan requirement

Open symbols indicate diets produced by 'diet dilution' technique, closed symbols indicate those produced by the 'graded supplementation' method

Diets I-III formulated by the method of graded supplementation
Diets V-VIII formulated by the method of diet dilution

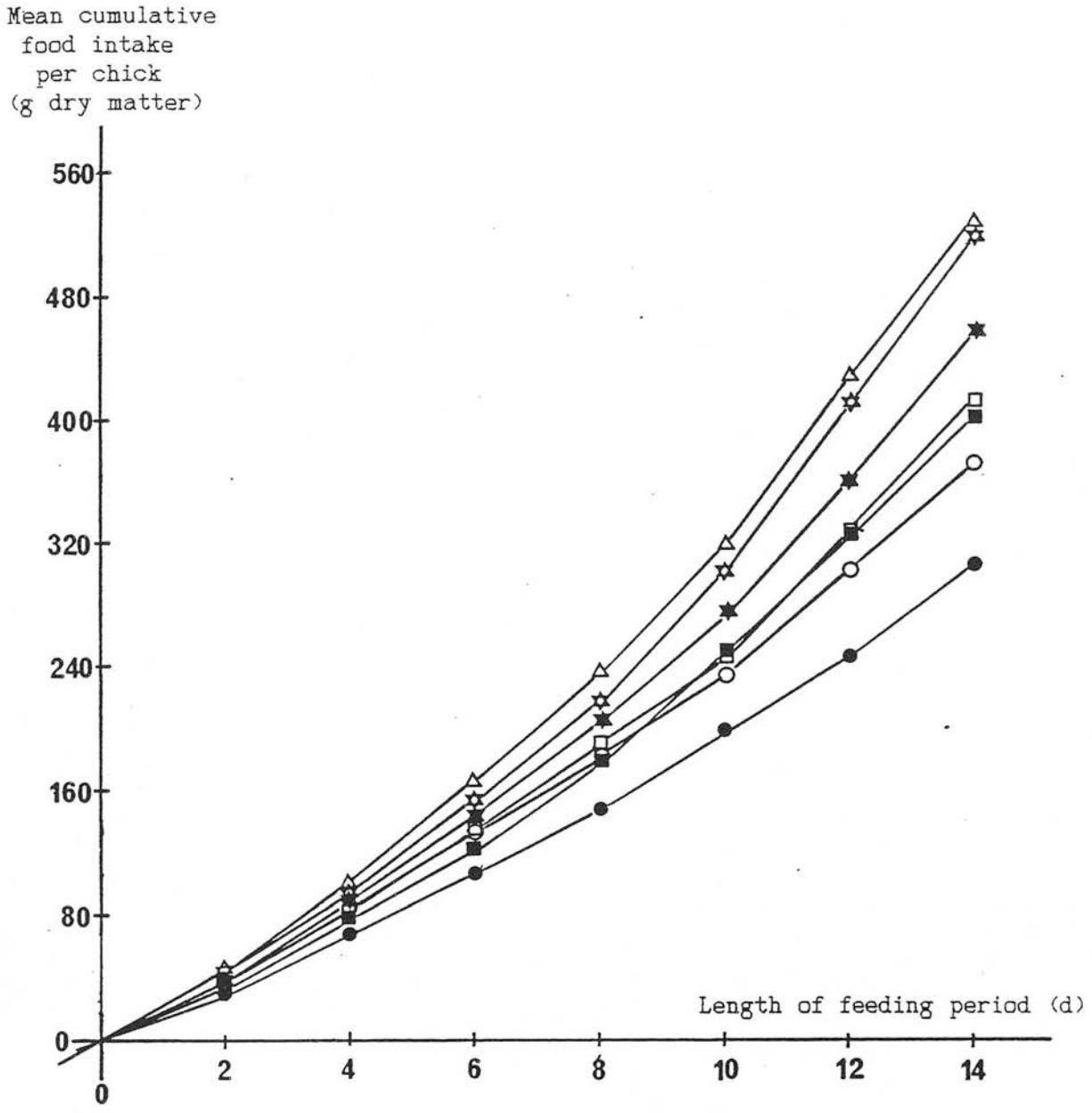


Fig. 21. Food intake of chicks fed diets differing in their amino acid balance and tryptophan content.

● ○ I and V, formulated to provide 0.48 of tryptophan requirement of the chick
■ □ II and VI, to provide 0.63 of tryptophan requirement
✱ ✡ III and VII, to provide 0.78 of tryptophan requirement
△ VIII, to provide total tryptophan requirement

Open symbols indicate diets produced by 'diet dilution' technique, closed symbols indicate those produced by the 'graded supplementation' method

Diets I-III formulated by the method of graded supplementation
Diets V-VIII formulated by the method of diet dilution

significantly greater weight gain on the second and the tenth day of the feeding period ($P<0.05$), food intake being increased only on day two ($P<0.05$).

The weight gain of chicks fed the diet supplemented with tryptophan at 0.63 of its required concentration was not significantly different from that of birds receiving the diluted diet providing the same amount of this amino acid. Only on the second day of the feeding period was the food intake of chicks fed the former diet less than that of those fed the latter ($P<0.05$).

The supplemented diet containing 0.78 of the required tryptophan concentration supported a greater chick weight gain and food intake than that containing 0.48 of the amount required ($P<0.001$). The weight gain of chicks fed the former diet was also significantly above that of those receiving the diluted diet containing 0.48 of the required amount of tryptophan ($P<0.001$). A significantly greater food intake of those fed the greater amount of tryptophan was not however observed until the tenth day of the feeding period ($P<0.05$), the difference having increased significance on the final day of the experiment ($P<0.01$).

Chicks consuming the supplemented diet incorporating 0.78 of the required amount of tryptophan also showed a greater gain in weight than those fed the diet of the same series containing 0.63 of that amount required. The difference was significant at a very high level on the second and sixth days of the feeding period ($P<0.001$) and at a somewhat lower level at all other times of measurement ($P<0.01$). The food intake of birds fed the greater amount of tryptophan was increased significantly only on the second ($P<0.01$), fourth ($P<0.05$) and sixth ($P<0.01$) days of the experimental period, and was not significantly different thereafter. In comparison with birds receiving

the diluted diet containing 0.63 of the required quantity of tryptophan, those fed the supplemented diet incorporating 0.78 of the required amount had a greater weight gain which was significant at the level of $P<0.001$ initially and at $P<0.01$ from day eight onwards. The food intake of chicks fed each of these diets was not significantly different.

The supplemented diet supplying tryptophan at 0.78 of its required concentration supported a chick weight gain which was not significantly different from that supported by the diluted diet providing the same amount of this amino acid. The food intake of chicks fed the former diet was not significantly different on days six, ten and twelve of the feeding period, but was significantly less than that of those fed the diluted diet at all other times of measurement ($P<0.05$).

Apart from the trends in food intake and weight gain already mentioned, several others were observable. The diluted diet providing 0.48 of the required amount of tryptophan for the chick supported a weight gain which was significantly below that of birds fed the diet of the same series supplying 0.63 of the tryptophan requirement only on the final day of the feeding period ($P<0.05$). The food intake of the chicks fed these diets was never significantly different. In comparison with chicks fed the diluted diet containing 0.78 of the required amount of tryptophan, those fed the diluted diet providing 0.48 of the requirement showed a lower weight gain ($P<0.001$). The food intake of chicks fed the latter diet was immediately reduced below that of birds consuming the former ($P<0.01$). The level of significance of this difference in food intake had fallen by the eighth day of feeding ($P<0.05$), but was restored on the tenth and increased on the twelfth and forteenth days of the experiment ($P<0.001$).

The weight gain of chicks fed the diluted diet incorporating 0.48 of the tryptophan requirement was very greatly below that of those consuming the tryptophan-adequate diluted diet ($P<0.001$). Food intake was correspondingly depressed on the second day of feeding ($P<0.01$) and thereafter ($P<0.001$).

By the second day of feeding, chicks receiving the diluted diet supplying 0.63 of the required amount of tryptophan showed a significantly greater weight gain than those fed the supplemented diet providing 0.48 of the requirement ($P<0.01$). The level of significance of this difference in weight gain was reduced only on the eighth day of the experimental period ($P<0.05$), and was increased on the final day ($P<0.001$). Food intake of birds fed the lower amount of tryptophan was less than that of those fed the greater amount. The difference was highly significant initially ($P<0.001$) but attained a lower level on the sixth and eighth ($P<0.01$) and tenth ($P<0.05$) days of the experiment. On the twelfth day of feeding the level of significance increased once more ($P<0.01$) and was at its highest at the end of the experiment ($P<0.001$).

Birds consuming the diluted diet providing 0.63 of the tryptophan requirement of the chick also had a lower weight gain than those fed 0.78 of the amount required, the difference being very highly significant ($P<0.001$) except on the sixth and eighth days of feeding ($P<0.01$). The food intake of birds fed the former diet was also below that of those receiving the latter, being significant at the level of $P<0.01$ apart from on the sixth and eighth days of feeding ($P<0.05$) and at the end of the experiment ($P<0.001$). In comparison with birds fed the tryptophan-adequate diluted diet, the weight gain of those fed the diet of the same series containing 0.63 of the tryptophan requirement was constantly and severely depressed ($P<0.001$). The food intake of chicks

fed the latter diet was correspondingly reduced, the depression being very highly significant on the fourth, sixth, twelfth and fourteenth days of feeding ($P<0.001$) and slightly less so at all other times ($P<0.01$).

The diluted diet providing 0.78 of the chick's tryptophan requirement supported a level of chick growth and food intake very much greater than that supported by the supplemented diet containing 0.48 of the tryptophan requirement. The significance of the differences in these measurements were at the level of $P<0.001$, apart from that of the difference in food intake on the eighth day of feeding ($P<0.01$). Chicks fed the supplemented diet providing 0.63 of their tryptophan requirement also showed a significantly lower weight gain and food intake than those receiving the diluted diet supplying 0.78 of the required amount. The difference in weight gain was significant at the level of $P<0.01$ initially and very highly significant on and after the tenth day of feeding ($P<0.001$). The food intake of birds fed these two diets was very significantly different ($P<0.001$) on all but the tenth and twelfth days ($P<0.01$) of the experimental period. In comparison with birds fed the tryptophan-adequate diluted diet, the weight gain of those consuming the diluted diet containing 0.78 of the required amount of tryptophan was significantly less initially ($P<0.01$) and on the fourth and tenth ($P<0.05$) days of feeding. Food intake of the groups of birds fed these two diets was not significantly different.

Both the weight gain and food intake of chicks fed the tryptophan-adequate diluted diet were constantly very significantly greater than those measured for birds fed the diet of the supplemented series providing 0.48 of the tryptophan requirement ($P<0.001$). Similarly, chicks receiving the supplemented diet supplying 0.63 of their tryptophan requirement had a weight gain below that of those fed the

diluted diet having an adequate content of this amino acid ($P<0.001$). The food intake of birds fed the former diet was also below that of those receiving the latter, the difference being very highly significant ($P<0.001$) on all but the tenth ($P<0.01$) day of feeding. Chicks receiving the supplemented diet containing 0.78 of their tryptophan requirement had a weight gain which was significantly less than that of birds fed the tryptophan-adequate diluted diet on the second and fourth ($P<0.05$), tenth ($P<0.01$) and fourteenth ($P<0.05$) days of the feeding period. The food intake of birds receiving the former diet was significantly less than that of those fed the tryptophan-adequate diluted diet ($P<0.05$), the significance of the difference in food intake attaining a higher level only on the fourth day of feeding ($P<0.01$).

Consideration of the variation in daily weight gain of the chicks with daily tryptophan intake (Fig. 22) indicates that there was little difference in the response to tryptophan of chicks consuming the diluted diets and those receiving the supplemented series.

d. Neurotransmitter concentrations

i. Dietary effects

Response ratios calculated indicated likely contamination of the DOPAC and HVA eluted from the HPLC column and these measurements were subsequently omitted from statistical analysis. As shown in Table 19, no significant differences in the brain concentrations of NE were observed. In chicks receiving the supplemented diet providing 0.78 of their tryptophan requirement, brain concentrations of E were depressed in comparison with those of chicks fed the supplemented diets providing 0.48 and 0.63 of the requirement ($P<0.05$), and of birds receiving the diluted diets formulated to be adequate in its tryptophan content ($P<0.01$). A significant depression in the brain concentration of DA in

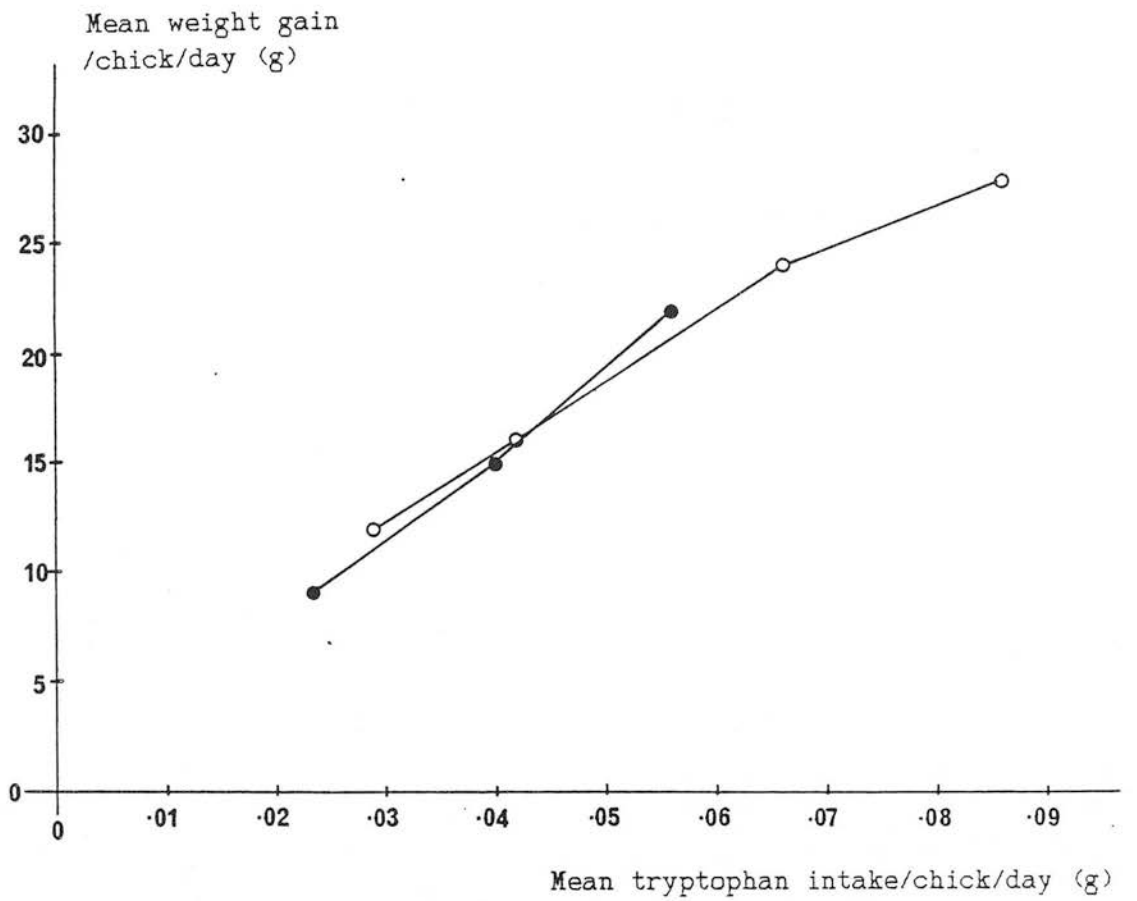


Fig. 22. Variation in daily weight gain with daily tryptophan intake for chicks fed diets differing in their amino acid balance and tryptophan content.

Open symbols indicate diets produced by 'diet dilution' technique, closed symbols indicate those produced by the 'graded supplementation' method

Table 19. Brain neurotransmitter concentrations of chick fed diets differing in their amino acid balance and tryptophan content.

Diet fed	Mean concentration of compound (ng/g fresh wt. brain tissue)				
	NE	E	DA	5HT	5HIAA
I	306	22	335	447	31
II	301	23	322	464	28
III	287	13*	251	498	52
V	288	19	301	505	45
VI	248	18	232	352	38
VII	245	16	228*	411	45
VIII	329	26	284	832***	87**
sem (21 d.f.)	29	3	36	66	12

Values significantly different from those obtained for chicks fed diet I
*P<0.05, **P<0.01, ***P<0.001

I and V, formulated to provide 0.48 of tryptophan requirement
II and VI, to provide 0.63 of tryptophan requirement
III and VII, to provide 0.78 of tryptophan requirement
VIII, to provide total tryptophan requirement

Diets I-III formulated by the 'graded supplementation' method, diets V-VIII by the 'diet dilution' method.

chicks fed the diluted diet providing 0.78 of the required amount of tryptophan, below that of those receiving the supplemented diet providing 0.48 of the requirement was also recorded ($P<0.05$).

Chicks receiving the diluted diet providing an adequate amount of tryptophan had a significantly higher brain concentration of 5HT than those fed diluted diets providing 0.78 or 0.63 ($P<0.001$) or 0.48 ($P<0.01$) of the estimated tryptophan requirement. Chicks receiving supplemented diets providing 0.48 and 0.63 of their estimated tryptophan requirement also had significantly lower brain concentrations of 5HT than those consuming the tryptophan-adequate diluted diet ($P<0.001$), as did birds fed the supplemented diet containing tryptophan at 0.78 of its requirement ($P<0.01$).

Birds receiving the tryptophan-adequate diet of the diluted series had a greater brain 5HIAA concentration than those fed diluted diets containing 0.48 or 0.78 ($P<0.05$) or 0.63 ($P<0.01$) of the required amount of this amino acid. The brain concentration of 5HIAA of birds fed the tryptophan-adequate diluted diet was also above that of those fed supplemented diets containing 0.48 or 0.63 of the tryptophan requirement ($P<0.01$.).

ii. Neurotransmitter concentrations and food intake

The brain concentrations of NE, DA and 5HT in chicks receiving the diets of this experiment (diet IV being excluded) were not significantly correlated with their intake of food as a whole or protein or AME(N) in particular. No correlation was apparent between the brain concentrations of NE or DA and the combined intake of tyrosine+ phenylalanine. However, the chick brain concentration of 5HT was significantly and positively correlated with the amount of tryptophan consumed ($r=0.455$, $P<0.05$).

3.7. Experiment 6. Zinc deficiency in the chick and an amino acid imbalance with respect to tyrosine and phenylalanine

a. Dietary zinc content

Diets formulated so as to be zinc-deficient were found to have a mean content of 14mg zinc and the zinc-adequate diets a mean of 71mg zinc per kg dietary dry matter.

b. Dietary nitrogen and AME(N) content

It appears from the contents of nitrogen and AME(N) determined for the experimental diets (Table 20.) that the low-protein control diet in the absence or presence of zinc had a much lower nitrogen content than all the other diets, which did not differ in nitrogen content by more than 2g/kg.

The AME(N) of the diet containing both the imbalancing amino acid mixture and a supplement of phenylalanine at 5g/kg was significantly lower than that of the fully supplemented diet ($P<0.001$) and all others except the zinc-deficient control diet ($P<0.05$). That of the fully-supplemented diet was also significantly greater than that of the zinc-supplemented control diet, the zinc-deficient diets containing the imbalancing amino acid mixture alone or with additional phenylalanine at a concentration of 10g/kg, and the zinc-adequate diet incorporating both the amino acid mixture and phenylalanine at 5g/kg ($P<0.05$).

c. Growth and food intake (Figs. 23 and 24.)

The low protein control diet, deficient in zinc, tyrosine and phenylalanine, supported a poor growth and food intake which was not significantly different from that supported by the zinc-adequate form. Chicks fed the zinc-deficient diet containing the imbalancing amino acid mixture showed immediate reductions in both growth and food intake below those of birds fed either the zinc-deficient or the zinc-

Table 20. Nitrogen and AME(N) content of diets formulated to study the effect of zinc deficiency on a tyrosine+phenylalanine imbalance

	DIET FED							
	I	II	III	IV	V	VI	VII	VIII
NITROGEN CONTENT(g/kg dry matter)	19.83	19.69	33.90	34.73	35.33	34.99	35.43	36.72
AME(N) (MJ/kg dry matter)	14.45	14.54	14.57	14.41	14.46	13.88	15.01	14.46

sem (21 d.f.)=0.18

Values of AME(N) content are not significantly different from that of the zinc-deficient control diet.

I	Zinc-deficient low-protein control diet with low content of tyrosine+ phenylalanine
II	Zinc-adequate version of control diet
III	Zinc-deficient low-protein diet+imbalancing amino acid mixture
IV	Zinc-adequate low-protein diet+imbalancing amino acid mixture
V	Zinc-deficient imbalanced diet+phenylalanine (5g/kg)
VI	Zinc-adequate imbalanced diet+phenylalanine (5g/kg)
VII	Zinc-deficient imbalanced diet+phenylalanine (10g/kg)
VIII	Zinc-adequate imbalanced diet+phenylalanine (10g/kg)

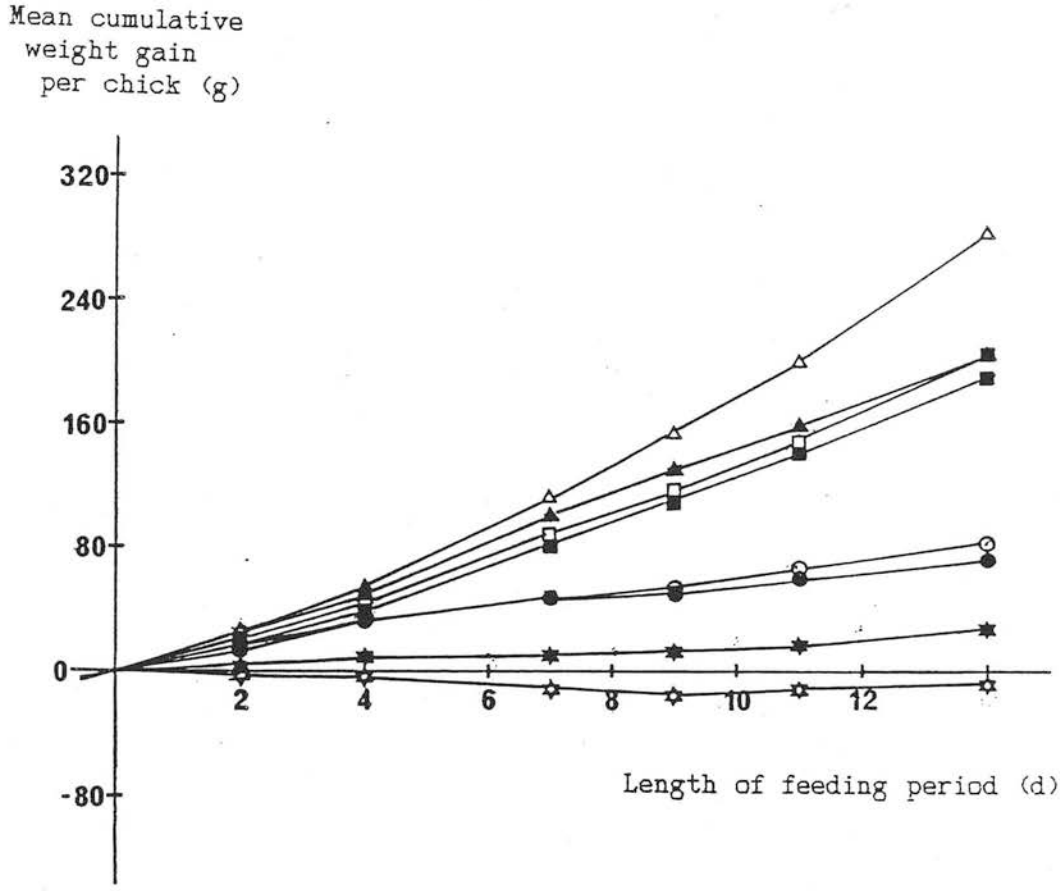


Fig. 23. Weight gain of chicks during study of the effect of zinc deficiency on a tyrosine+phenylalanine imbalance

- ● I Zinc-deficient low-protein control diet with low content of tyrosine+ phenylalanine
- ○ II Zinc-adequate version of control diet
- ✱ III Zinc-deficient low-protein diet+imbalancing amino acid mixture
- ✡ IV Zinc-adequate low-protein diet+imbalancing amino acid mixture
- ■ V Zinc-deficient imbalanced diet+phenylalanine (5g/kg)
- □ VI Zinc-adequate imbalanced diet+phenylalanine (5g/kg)
- ▲ VII Zinc-deficient imbalanced diet+phenylalanine (10g/kg)
- △ VIII Zinc-adequate imbalanced diet+phenylalanine (10g/kg)

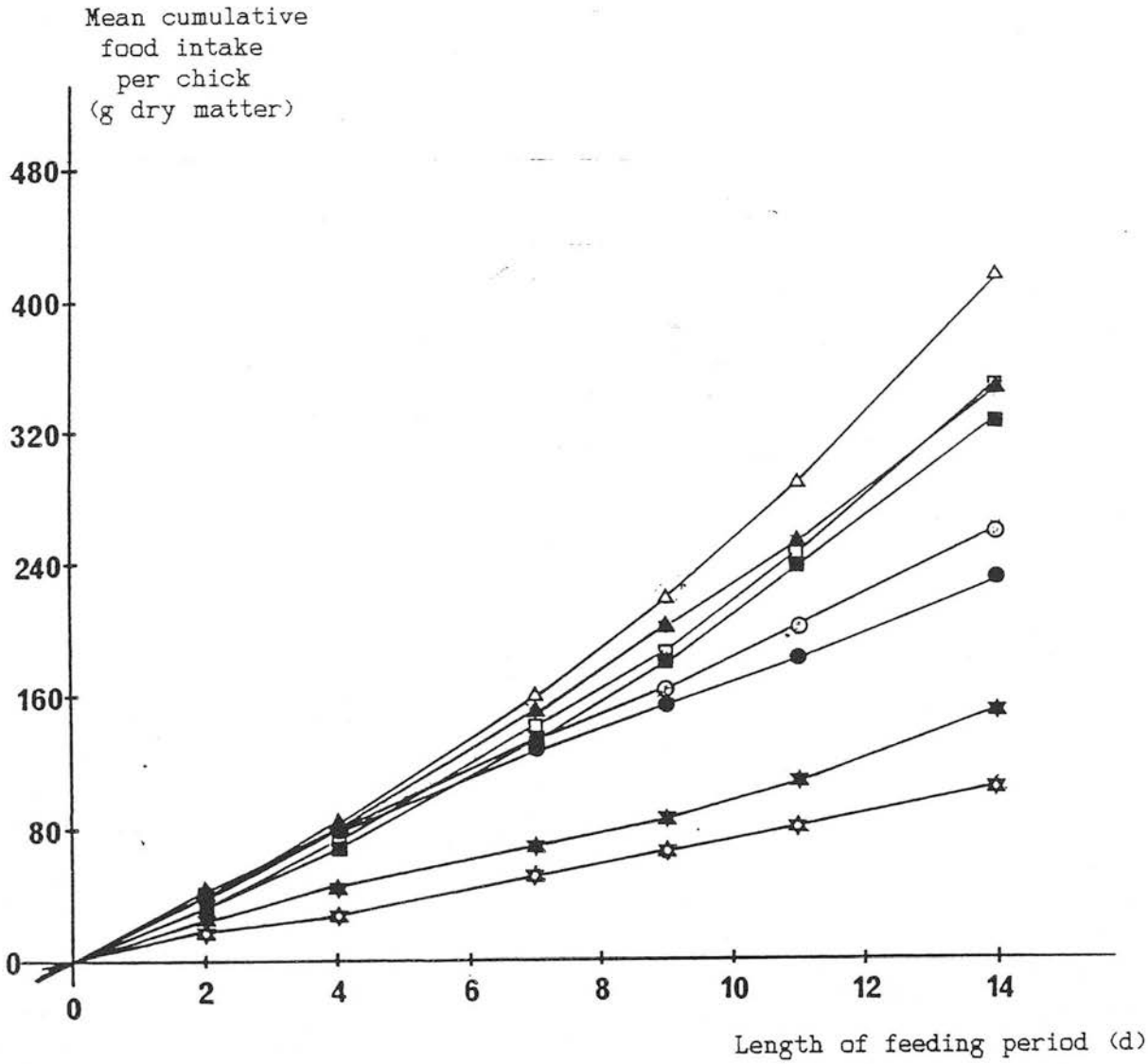


Fig. 24. Food intake of chicks during study of the effect of zinc deficiency on a tyrosine+phenylalanine imbalance

- ● I Zinc-deficient low-protein control diet with low content of tyrosine+ phenylalanine
- ○ II Zinc-adequate version of control diet
- ✱ III Zinc-deficient low-protein diet+imbalancing amino acid mixture
- ✡ IV Zinc-adequate low-protein diet+imbalancing amino acid mixture
- ■ V Zinc-deficient imbalanced diet+phenylalanine (5g/kg)
- □ VI Zinc-adequate imbalanced diet+phenylalanine (5g/kg)
- ▲ VII Zinc-deficient imbalanced diet+phenylalanine (10g/kg)
- △ VIII Zinc-adequate imbalanced diet+phenylalanine (10g/kg)

adequate control diet ($P<0.001$). Those chicks consuming the comparable zinc-adequate imbalanced diet also had levels of growth and food intake below those of birds fed the control diet in the presence or absence of zinc supplementation ($P<0.001$). However in this case a mean weight loss was recorded and the difference in weight gain between chicks fed the zinc-deficient and zinc-adequate imbalanced diets was initially highly significant ($P<0.001$). This degree of significance had fallen by the seventh day of feeding ($P<0.01$) and was further decreased on day eleven ($P<0.05$). By the final day of the experimental period there was no longer a difference in weight gain observed between chicks fed these diets. Intake of the zinc-adequate imbalanced diet was depressed in comparison with that of its zinc-deficient counterpart on the second ($P<0.05$), fourth ($P<0.001$), seventh ($P<0.01$), ninth and eleventh ($P<0.05$) days of the feeding period, and also at the end of the experiment ($P<0.01$).

Regardless of the dietary zinc content, incorporation of a supplement of phenylalanine at a level of 5g/kg into the imbalanced diets improved chick growth and food intake to a level above that supported by diets containing the imbalancing amino acid mixture alone ($P<0.001$). The weight gain of chicks fed the zinc-deficient diet incorporating both the imbalancing mixture and this phenylalanine supplement was increased above that of those fed the control diet alone or with a supplement of zinc on, and subsequent to, the seventh day of feeding ($P<0.001$). Food intake of chicks fed the former diet initially was reduced below that of birds fed the control diet alone or with additional zinc ($P<0.001$). The level of significance of this depression in food intake below that of birds fed the zinc-deficient control diet was reduced by the fourth day of feeding ($P<0.01$). By day seven, the

food intake was completely restored to that of birds fed the control diet alone or with zinc, and on day nine was significantly increased ($P<0.01$). The increase in food intake above that of birds fed the zinc-deficient control diet subsequently attained significance at the level of $P<0.001$. Only on the final day of the experiment was the difference in food intake between chicks fed the zinc-supplemented control diet and those receiving the zinc-deficient diet incorporating the imbalancing amino acid mixture and phenylalanine at 5g/kg significant at this level ($P<0.001$).

The zinc-adequate diet incorporating the imbalancing amino acid mixture and phenylalanine at 5g/kg constantly supported greater levels of weight gain and food intake in the chick than either the zinc-deficient or the zinc-adequate imbalanced diet ($P<0.001$). Birds fed this diet also were shown after two days of feeding to have a significantly greater weight gain than those fed the zinc-supplemented control diet ($P<0.05$). From day four onwards, the weight gain of chicks fed the former diet was very significantly greater than that of those fed the control diet alone or with a zinc supplement ($P<0.001$). Food intake of chicks receiving the zinc-adequate diet supplemented with the amino acid mixture and phenylalanine at 5g/kg was initially lower than that of those fed the control diet alone or with zinc ($P<0.001$). By day four, food intake had been restored to a level comparable to that of birds fed the unsupplemented control diet and was significantly different from that of those receiving the zinc-supplemented control diet at a much reduced level ($P<0.05$). On the seventh day of feeding, chicks fed the diet supplemented with zinc, the amino acid mixture and phenylalanine at 5g/kg had a level of food intake which was above that of those fed the unsupplemented control diet ($P<0.05$) but not

significantly different from that of birds fed the zinc-supplemented control diet. By day nine, the increase in food intake relative to that of chicks fed the unsupplemented control diet had attained greater significance ($P<0.01$), while the increase in food intake relative to that of chicks fed the zinc-adequate control diet reached significance at the level of $P<0.05$. On the eleventh day of the feeding period these differences had reached levels of significance of $P<0.001$ and $P<0.01$ respectively, and were both very highly significant at the end of the experiment ($P<0.001$).

Comparison of chicks fed the zinc-adequate diet containing the amino acid mixture and supplemental phenylalanine at 5g/kg with those consuming its zinc-deficient counterpart showed differences only on day four, when weight gain of chicks fed the zinc-deficient version was significantly lower ($P<0.05$).

Chicks fed either of the diets incorporating the imbalancing amino acid mixture and a supplement of phenylalanine at a concentration of 10g/kg showed much greater levels of growth and food intake than birds fed either of the diets containing the imbalancing amino acid mixture without additional phenylalanine ($P<0.001$). Raising the level of phenylalanine supplementation to 10g/kg also gave an immediate improvement in weight gain above that of chicks fed the zinc-supplemented control diet ($P<0.001$). The improvement in comparison with birds fed the zinc deficient control diet was initially less significant ($P<0.01$), but was very highly significant by the fourth day of feeding ($P<0.001$). Food intake showed the same trend as described previously, intake of the zinc-deficient diet supplemented with the amino acid mixture and phenylalanine at 10g/kg being initially below that of the control diet alone ($P<0.05$) or supplemented with zinc ($P<0.01$). This was

followed by the restoration of food intake to that of the control diet with or without zinc by day four, and its rise by day seven above that of the zinc-deficient ($P<0.001$) or zinc-supplemented ($P<0.01$) control diets. The increase in food intake above that of the control diet alone or with zinc was very highly significant thereafter ($P<0.001$). Chicks fed the zinc-adequate, fully supplemented diet showed an initial level of food intake which was not significantly different from that of the zinc-deficient control diet but was below that of the control diet supplemented with zinc ($P<0.05$). Food intake was restored by the fourth day of feeding and subsequently was increased above that of birds fed the control diet in the presence or absence of zinc ($P<0.001$).

Chicks fed the zinc-deficient diet incorporating both the imbalancing amino acid mixture and supplemental phenylalanine at 10g/kg showed a further increase in growth above that observed with the lesser phenylalanine supplement in the absence of zinc, this being significant initially ($P<0.01$) and on the fourth ($P<0.001$), seventh ($P<0.01$) and ninth ($P<0.05$) days of feeding. The significance of this improvement was however lost by the eleventh day of the experimental period. Only on the seventh day of feeding did birds fed the zinc-deficient diet containing both the amino acid mixture and phenylalanine at 10g/kg have a significantly greater weight gain than those fed the zinc-adequate diet supplemented with phenylalanine at 5g/kg ($P<0.05$).

The food intake of birds fed the zinc-deficient diet containing the larger supplement of phenylalanine was greater than that of those receiving half the amount of this amino acid in the absence of zinc, the difference being significant at the level of $P<0.01$ initially, but at $P<0.05$ on day nine and no longer significant thereafter. Food intake was only increased above that of those receiving the zinc-

adequate diet containing the smaller phenylalanine supplement ($P<0.01$) on the second day of feeding.

On the second day of the feeding period, chicks consuming the zinc-adequate form of the diet supplemented with the imbalancing amino acid mixture and phenylalanine at 10g/kg had a greater weight gain and food intake than those fed the zinc-deficient diet containing the lower phenylalanine supplement ($P<0.01$). These differences were subsequently very highly significant ($P<0.001$). Chicks fed the fully-supplemented diet also had a larger gain in weight than that measured for those receiving the control diet supplemented with zinc on the fourth day of feeding ($P<0.01$) and thereafter ($P<0.001$). The food intake of birds receiving the former diet was significantly above that of those fed the latter on the second ($P<0.01$), fourth ($P<0.05$), seventh ($P<0.01$), ninth ($P<0.001$), eleventh ($P<0.01$) and fourteenth ($P<0.001$) days of the experimental period.

By the ninth day of feeding, the weight gain and food intake of chicks fed the zinc-adequate diet incorporating phenylalanine at 10g/kg was greater than that of those fed the zinc-deficient version of this diet ($P<0.05$), the difference having increased in significance by day eleven ($P<0.01$) and being very highly significant by the end of the experimental period ($P<0.001$).

d. Neurotransmitter concentrations

i. Dietary effects

Epinephrine was undetectable in these samples, while concentrations of HVA were so low that intra-sample variation was unsatisfactory and the results were disregarded. As shown in Table 21, the brain NE or DA concentrations of chicks fed the zinc-adequate control diet were not significantly different from those of birds

Table 21. Concentrations of neurotransmitters and metabolites in the chick brain during study of the effect of zinc deficiency on a tyrosine+phenylalanine imbalance

Diet fed	Concentration of compound (ng/g fresh wt. brain tissue) NE	DA	DOPAC	5HT	5HIAA
I	131	212	149	549	126
II	200	290	151	510	170
III	109	159	81	475	95
IV	219*	286	131	403	286***
V	166	180	90	514	180
VI	218	293	156	500	293***
VII	174	198	70	557	88
VIII	290***	365**	164	555	139
sem (21 d.f.)	28	38	29	25	76

Values significantly different from those determined in chicks fed zinc-deficient control diet *P<0.05, **P<0.01, ***P<0.001

I	Zinc-deficient low-protein control diet with low content of tyrosine+ phenylalanine
II	Zinc-adequate version of control diet
III	Zinc-deficient low-protein diet+imbalancing amino acid mixture
IV	Zinc-adequate low-protein diet+imbalancing amino acid mixture
V	Zinc-deficient imbalanced diet+phenylalanine (5g/kg)
VI	Zinc-adequate imbalanced diet+phenylalanine (5g/kg)
VII	Zinc-deficient imbalanced diet+phenylalanine (10g/kg)
VIII	Zinc-adequate imbalanced diet+phenylalanine (10g/kg)

receiving its zinc-deficient counterpart. Addition of the imbalancing amino acid mixture resulted in birds fed the zinc-supplemented imbalanced diet lacking phenylalanine supplementation having a significantly greater brain concentration of NE than those receiving the zinc-deficient control or imbalanced diets ($P<0.05$). Chicks fed the zinc-deficient imbalanced diet had a significantly lower brain NE concentration than those fed the zinc-adequate control diet ($P<0.05$). The brain DA concentration of these chicks was also significantly below that of those fed the zinc-adequate control or imbalanced diets ($P<0.05$). No significant difference in brain 5HT concentrations of chicks fed the control or imbalanced diets was observed, however those receiving the zinc-adequate control diet were shown to have higher brain concentrations of 5HIAA than birds fed the zinc-deficient imbalanced diet ($P<0.05$). Chicks receiving the zinc-adequate imbalanced diet had greater brain 5HIAA concentrations than those fed the zinc-deficient control diet ($P<0.001$) or either of the imbalanced diets, whether deficient ($P<0.001$) or adequate ($P<0.01$) in zinc content.

Supplementation of the imbalanced diets with phenylalanine at a concentration of 5g/kg appeared to have little effect, the brain NE and DA concentrations of birds receiving the zinc-adequate form of this diet however, being significantly higher than that of those consuming the zinc-deficient imbalanced diet ($P<0.05$). The concentration of DA was also greater than that measured in chicks fed the zinc-deficient form of the diet containing the amino acid mixture and phenylalanine at 5g/kg ($P<0.05$). No significant changes in the brain concentration of 5HT were apparent. Birds fed the zinc-deficient diet containing both the imbalancing amino acid mixture and phenylalanine at a concentration of 5g/kg had a lower brain concentration of DA than those receiving the

control diet supplemented with zinc ($P<0.01$). They also showed greater concentrations of 5HIAA than birds fed the zinc-deficient imbalanced diet ($P<0.05$). This concentration remained however significantly below that of chicks fed the zinc-adequate control diet ($P<0.01$) or the zinc-adequate diet supplemented with the same amount of phenylalanine ($P<0.01$). Chicks consuming the latter also had significantly higher brain concentrations of 5HIAA than those fed the zinc-deficient ($P<0.001$) or zinc-adequate ($P<0.01$) control diets or zinc-deficient imbalanced diet ($P<0.001$).

At the highest level of supplementation with phenylalanine to correct the zinc-adequate imbalanced diet, chick brain concentrations of NE and DA were significantly increased above those determined in birds fed the zinc-deficient control diet ($P<0.001$ and $P<0.01$ respectively). The brain concentration of NE was also greater than that of birds fed the zinc-supplemented control diet ($P<0.05$). Both NE and DA concentrations in the brains of chicks fed this fully supplemented diet were significantly higher than in those receiving the zinc-deficient imbalanced diet ($P<0.001$) or the zinc-deficient diets incorporating the imbalancing amino acid mixture and either concentration of phenylalanine ($P<0.01$). The brain concentration of DOPAC was also greater than that of birds fed either of the zinc-deficient diets supplemented with phenylalanine ($P<0.05$). Concentrations of 5HT were again unaffected by feeding the diets. Brain concentrations of 5HIAA were significantly less in chicks receiving the zinc-adequate or deficient diets incorporating both the imbalancing amino acid mixture and the maximum incorporated concentration of phenylalanine in comparison with birds fed the zinc-adequate imbalanced diet lacking phenylalanine supplementation or supplemented with only 5g/kg ($P<0.001$). Chicks receiving the zinc-

deficient diet incorporating the imbalancing amino acid mixture and having maximum supplementation with phenylalanine also had a significantly lower brain concentration of 5HIAA than birds fed the zinc-supplemented control diet or that zinc-deficient diet supplemented with the smaller concentration of phenylalanine ($P<0.05$).

ii. Neurotransmitter concentrations and food intake

The brain concentrations of NE, DA and 5HT in chicks fed the diets of this investigation were not significantly correlated with the total food intake of the birds or the quantity of protein or AME(N) ingested by them. No correlation was apparent between the chick brain concentrations of NE or DA and the combined intake of tyrosine+ phenylalanine. Similarly, the brain concentration of 5HT was not significantly correlated with the quantity of tryptophan ingested.

3.8. Experiment 7. Effect of ephedrine on a dietary tyrosine+ phenylalanine imbalance

a. Dietary nitrogen and AME(N) content

The low-protein control diet alone or with incorporation of ephedrine had a much lower nitrogen content than any of the other diets, which differed from each other in nitrogen content by not more than 1.4g/kg (Table 22). The nitrogen contents of the ephedrine-lacking diets were similar to those measured for the identical (zinc-adequate) diets of the previous investigation.

The AME(N) contents of the ephedrine-containing but otherwise unsupplemented diet, and the fully-supplemented diet incorporating ephedrine, were significantly greater than that of the control diet ($P<0.05$). The diet into which both ephedrine and the indispensable amino acid mixture had been introduced had a lower AME(N) content than that incorporating only the drug ($P<0.01$), while that

Table 22. Dietary nitrogen and AME(N) contents during investigation of the effects of ephedrine on the response of the chick to a tyrosine+phenylalanine imbalance

	DIET FED					
	I	II	III	IV	V	VI
NITROGEN CONTENT (g/kg dry matter)	19.05	19.13	34.45	34.78	35.07	35.80
AME(N) (MJ/kg dry matter)	14.45	14.86*	14.57	14.27	14.44	14.79*

sem (15 d.f.)=0.12

Values significantly different from that of control diet *P<0.05, **P<0.01

I Low-protein control diet having low content of tyrosine and phenylalanine
II Control+ephedrine (10g/kg diet)
III Control+amino acid mixture lacking tyrosine and phenylalanine
IV Control+ephedrine+amino acid mixture lacking tyrosine and phenylalanine
V Control+imbalancing amino acid mixture+phenylalanine (10g/kg diet)
VI Control+ephedrine+imbalancing amino acid mixture+phenylalanine (10g/kg diet)

supplemented with both the indispensable amino acid mixture and additional phenylalanine but lacking ephedrine had a significantly lower AME(N) than the identical diet containing the drug or the drug-containing diet lacking amino acid supplements ($P<0.05$).

b. Growth and food intake (Figs. 25 and 26)

Chicks consuming the control diet supplemented with ephedrine immediately had significantly reduced levels of weight gain and food intake relative to those fed the control diet alone ($P<0.001$). From day twelve onwards, the level of significance of the difference in weight gain of birds fed these two diets was reduced to $P<0.01$, the difference in food intake attaining significance only at this level onthe final day of the experimental period.

As in the previous investigation, addition of the imbalancing amino acid mixture to the control diet caused a depression in weight gain below that of birds fed the control diet alone, having significance at the level of $P<0.01$ by day two and at $P<0.001$ throughout the remainder of the feeding period. Food intake was consistently severely reduced ($P<0.001$). However no mean weight loss of birds receiving this diet was observed as had been seen for those fed the identical diet previously, although total mean food intake was similar. On the second day of feeding, the weight gain of birds fed this imbalanced diet was greater than that of chicks fed the ephedrine-supplemented control diet ($P<0.05$), but was not subsequently significantly different. Food intake of birds receiving the former diet was initially less than that of those consuming the latter ($P<0.01$) but was not significantly different on day four. Only on the twelfth and fourteenth days of feeding was the lower food intake of birds receiving

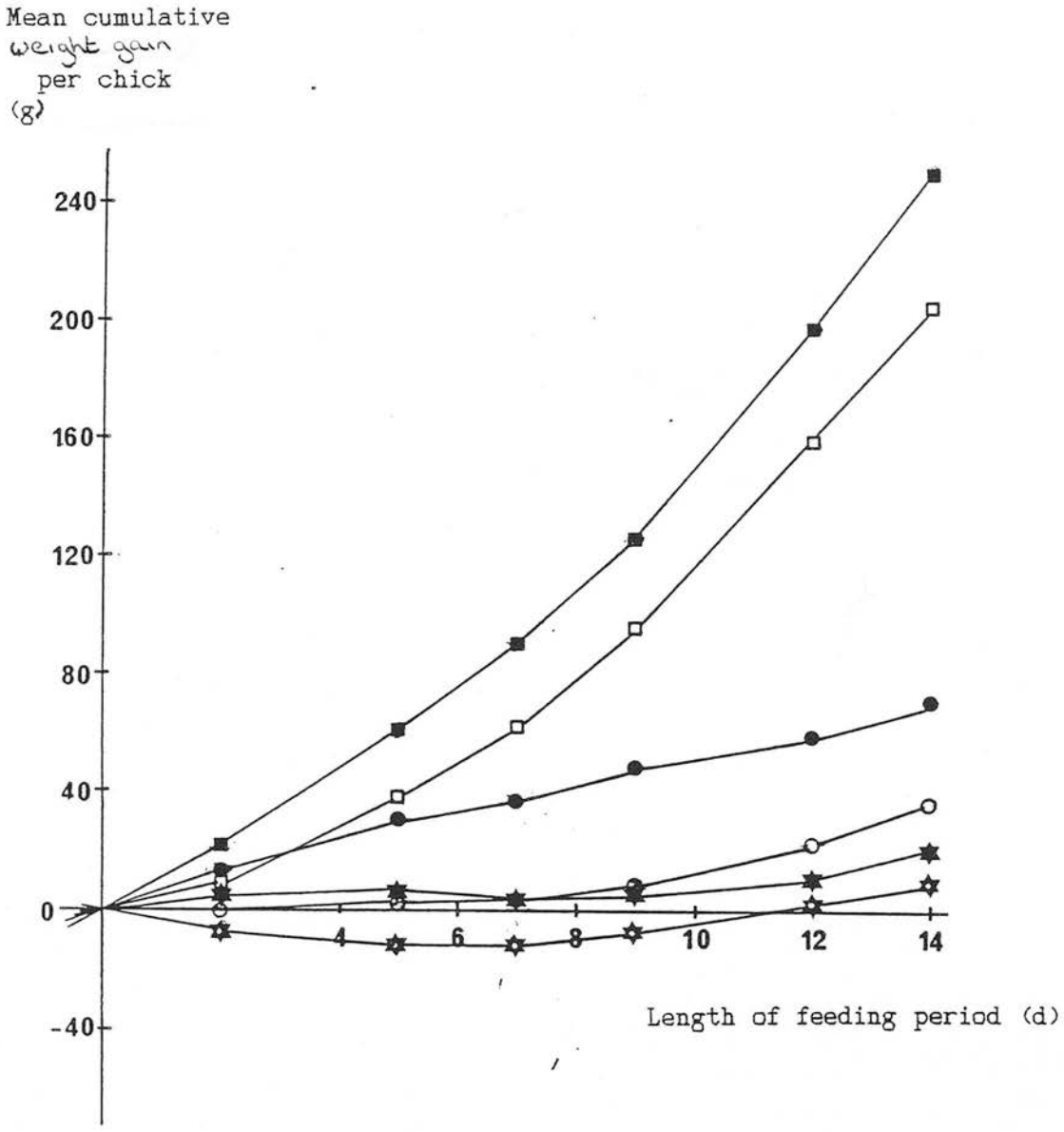


Fig 25: Weight gain of chicks during investigation of the effect of ephedrine on a dietary tyrosine+phenylalanine imbalance

- ● I Low-protein control diet having low content of tyrosine and phenylalanine
- ○ II Control+ephedrine (10g/kg diet)
- ✱ III Control+amino acid mixture lacking tyrosine and phenylalanine
- ✡ IV Control+ephedrine+amino acid mixture lacking tyrosine and phenylalanine
- ■ V Control+imbalancing amino acid mixture+phenylalanine (10g/kg diet)
- □ VI Control+ephedrine+imbalancing amino acid mixture+phenylalanine (10g/kg diet)

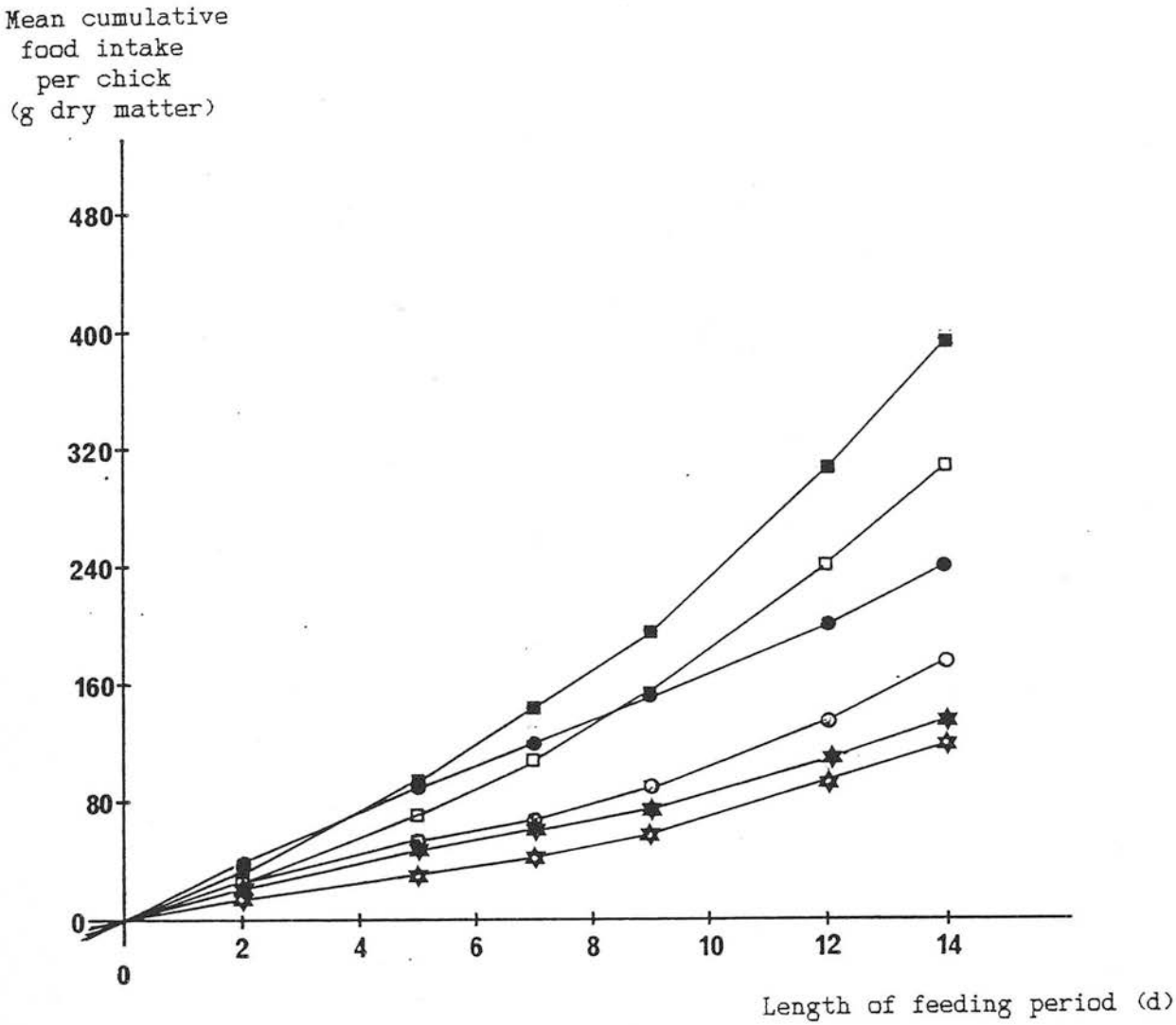


Fig 26. Food intake of chicks during investigation of the effect of ephedrine on a dietary tyrosine+phenylalanine imbalance

- ● I Low-protein control diet having low content of tyrosine and phenylalanine
- ○ II Control+ephedrine (10g/kg diet)
- ✱ III Control+amino acid mixture lacking tyrosine and phenylalanine
- ✲ IV Control+ephedrine+amino acid mixture lacking tyrosine and phenylalanine
- ■ V Control+imbalancing amino acid mixture+phenylalanine (10g/kg diet)
- □ VI Control+ephedrine+imbalancing amino acid mixture+phenylalanine (10g/kg diet)

the imbalanced diet again significantly different from that of those fed the drug-supplemented control diet ($P<0.05$).

Chicks consuming the imbalanced diet which was also supplemented with ephedrine at all times showed highly significant reductions in weight gain and food intake below those measured for birds fed the control diet ($P<0.001$). The weight gain of chicks fed the ephedrine-supplemented imbalanced diet was also below that of those receiving the control diet supplemented with this drug, the difference being significant at the level of $P<0.01$ initially and $P<0.05$ from the seventh day of feeding onwards. A depression in food intake accompanied this reduction in weight gain and was very highly significant ($P<0.001$) until the twelfth day of feeding, significance being attained at the level of $P<0.01$ thereafter.

The ephedrine-containing imbalanced diet supported a weight gain which was immediately highly significantly below that supported by the identical diet in the absence of the drug ($P<0.001$). However the degree of significance of this depression fell by day five ($P<0.01$) and day seven ($P<0.05$), and by the twelfth day of feeding there was no significant difference in weight gain of birds fed each of the two diets. Food intake was similarly reduced initially ($P<0.001$), the level of significance of this reduction falling on the seventh ($P<0.01$) and ninth ($P<0.05$) days of feeding and no significant difference being observable on and subsequent to day twelve.

As with the previous investigation, an addition of phenylalanine at a level of 10g/kg to the imbalanced diet restored amino acid balance and improved growth and food intake. By the second day of feeding, weight gain of chicks consuming this diet showed an increase over that of those receiving the control diet ($P<0.01$), and thereafter

this increase was highly signifcant ($P<0.001$). Food intake followed a pattern similar to that seen for the same diets in the previous experiment; that is, an initial reduction in intake relative to the control diet ($P<0.01$) was followed by its restoration after five days of feeding and subsequent rise above control levels ($P<0.001$). Both the weight gain and food intake of chicks fed this phenylalanine-supplemented diet were constantly much greater than those of birds fed the ephedrine-supplemented control diet or the imbalanced diet alone or with ephedrine ($P<0.001$).

Chicks receiving the diet incorporating the amino acid mixture, additional phenylalanine and also a supplement of ephedrine initially had a lower weight gain than those fed the control diet ($P<0.05$). The significance of this reduction was lost by the fourth day of feeding and subsequently birds fed the former diet had a significantly greater weight gain than those fed the control diet ($P<0.001$). The food intake of chicks fed the diet supplemented with the amino acid mixture, phenylalanine and ephedrine was depressed below that of those fed the control diet until the seventh day of feeding ($P<0.001$) and was not increased above this level until days twelve ($P<0.01$) and fourteen ($P<0.001$). On the second day of the feeding period, birds receiving the ephedrine-supplemented control diet showed a significantly lower weight gain than those fed the drug-supplemented diet containing the amino acid mixture and phenylalanine ($P<0.05$), food intake of chicks fed these diets being not significantly different at this point. From the fourth day of feeding onwards, both weight gain and food intake of chicks fed the ephedrine-containing diet supplemented with the amino acid mixture and phenylalanine were above those of birds fed the drug-supplemented control diet ($P<0.001$). Chicks receiving the former diet

constantly showed levels of growth and food intake which were very much greater than those of birds fed the imbalanced diet alone or with ephedrine ($P<0.001$)

The effect of the addition of ephedrine to the diet incorporating the amino acid mixture and phenylalanine at 10g/kg was to reduce both weight gain and food intake below that supported by the same diet in the absence of the drug, a high degree of significance between the effects of these diets being maintained throughout the experimental period ($P<0.001$), with the exception of the difference in weight gain on the twelfth day of feeding, which attained significance at $P<0.01$.

c. Neurotransmitter concentrations

i. Dietary effects

Due to possible contamination problems, determined brain concentrations of HVA have been omitted from consideration. As indicated in Table 23, chicks fed the ephedrine-lacking diet in which the created imbalance had been corrected by addition of phenylalanine showed a significantly higher brain concentration of NE than those fed the totally unsupplemented diet ($P<0.05$), that incorporating only ephedrine ($P<0.01$), or the ephedrine-containing imbalanced or phenylalanine-supplemented diets ($P<0.05$). The brain concentration of E in this same group of birds was also significantly higher than in those receiving the low-protein, drug-containing diet lacking amino acid supplementation ($P<0.05$). The brain concentration of DA in chicks consuming the totally unsupplemented diet was found to be greatly above that of those fed all other diets ($P<0.001$). Birds receiving the diet containing both the imbalancing amino acid mixture and a phenylalanine supplement, but into which ephedrine had not been incorporated, showed significantly greater

Table 23. Brain concentrations of neurotransmitters and metabolites in chicks during investigation of the effect of ephedrine on a dietary tyrosine+phenylalanine imbalance

Diet fed	Mean concentration of compound (ng/g fresh wt. brain tissue) NE	E	DA	DOPAC	5HT	5HIAA
I	177	21	407	48	526	88
II	120	16	138***	39	584	96
III	209	23	195***	54	554	88
IV	156	25	131***	39	696	175*
V	283*	32	231***	47	628	80
VI	159	26	132***	34	487	100
sem (15 d.f.)	31	5	25	8	78	23

Values significantly different from control values *P<0.05, **P<0.01, ***P<0.001

I Low-protein control diet having low content of tyrosine and phenylalanine
II Control+ephedrine (10g/kg diet)
III Control+amino acid mixture lacking tyrosine and phenylalanine
IV Control+ephedrine+amino acid mixture lacking tyrosine and phenylalanine
V Control+imbalancing amino acid mixture+phenylalanine (10g/kg diet)
VI Control+ephedrine+imbalancing amino acid mixture+phenylalanine (10g/kg diet)

brain concentrations of DA than those fed any of the diets containing the drug ($P<0.05$). No significant differences in the brain concentrations of DOPAC in birds fed the various diets was found.

The concentration of 5HT in the brains of chicks fed each of the experimental diets did not differ significantly, while birds receiving the diet incorporating both the imbalancing amino acid mixture and ephedrine showed significantly higher brain concentrations of 5HIAA than those fed any of the other diets ($P<0.05$).

ii. Neurotransmitter concentrations and food intake

No significant correlations were apparent between the brain concentrations of NE, DA and 5HT in chicks fed the experimental diets and their total intake of food or the quantities of protein or AME(N) ingested by them. No correlation was apparent between the chick brain concentrations of NE or DA and the combined intake of tyrosine+phenylalanine, while the brain concentration of 5HT was not significantly correlated with the quantity of tryptophan consumed.

3.9. Experiment 8. The effects of increased incorporation of balanced and imbalanced proteins into the diet

a. Dietary nitrogen and AME(N) content

It appears from the determined nitrogenand AME(N) contents of the experimental diets fed during this investigation (Table 24) that the dietary nitrogen content increased with increasing incorporation of either casein or gelatin, those containing gelatin tending to have a somewhat greater content than those intended to provide an eqivalent amount of nitrogen from casein.

The AME(N) content of the diet containing gelatin to increase dietary nitrogen by 29g/kg was significantly lower than that of the unsupplemented control diet($P<0.01$) and diets containing an amount

Table 24. Variation in AME(N) and nitrogen content of diets containing different amounts of a balanced or an imbalanced protein

	DIET FED						
	I	II	III	IV	V	VI	VII
NITROGEN CONTENT (g/kg dry matter)	34.19	48.15	63.23	79.03	52.55	63.70	83.74
AME(N) (MJ/kg dry matter)	14.06	13.85	14.07	14.50	13.05	12.05**	10.33***

sem (18 d.f.)=0.46

Values significantly different from those of control diet *P<0.05, **P<0.01, ***P<0.001

I Control diet having nitrogen content of 33.28g/kg
II Control+casein equivalent of 16g nitrogen/kg diet
III Control+casein equivalent of 29g nitrogen/kg diet
IV Control+casein equivalent of 48g nitrogen/kg diet
V Control+gelatin equivalent of 16g nitrogen/kg diet
VI Control+gelatin equivalent of 29g nitrogen/kg diet
VII Control+gelatin equivalent of 48g nitrogen/kg diet

of casein equivalent to a nitrogen content of 16g/kg ($P<0.05$), 29g/kg ($P<0.01$) or 48g/kg ($P<0.01$). That diet incorporating an amount of gelatin equivalent to a nitrogen content of 48g/kg had an AME(N) content significantly lower than that of all these diets ($P<0.001$) and also lower than that of the diets incorporating gelatin at a concentration equivalent to a nitrogen content of 16g/kg ($P<0.001$) or 29g/kg ($P<0.05$).

b. Growth and food intake (Figs. 27 and 28)

Addition of casein to the control diet at a level equivalent to an increase in nitrogen content of 16g/kg gave no significant change in weight gain, food intake being reduced below that of the control diet on the second ($P<0.05$) and fourth ($P<0.01$) days of the feeding period, but restored by the sixth day. Increasing the casein level to that of 29g/kg with respect to dietary nitrogen also resulted in no change in weight gain. Food intake was initially reduced below that of the control diet ($P<0.001$), but after six days the levels of food intake of chicks fed these two diets were not significantly different from each other. Neither the weight gain nor the food intake of birds fed the diet incorporating casein at the level equivalent to a nitrogen content of 29g/kg was significantly different from that of chicks fed that containing half this amount of casein.

When casein was added to the control diet at a level causing an increase in ditary nitrogen content of 48g/kg, the observed weight gain was reduced by the fourth day of feeding compared with that of chicks receiving the control diet ($P<0.05$), the reduction attaining significance at the level of $P<0.01$ on and after day twelve. There was a continual, highly significant depression in food intake ($P<0.001$). The weight of chicks fed the greatest quantity of casein was also below that of those fed the diet containing this protein at its lowest level of

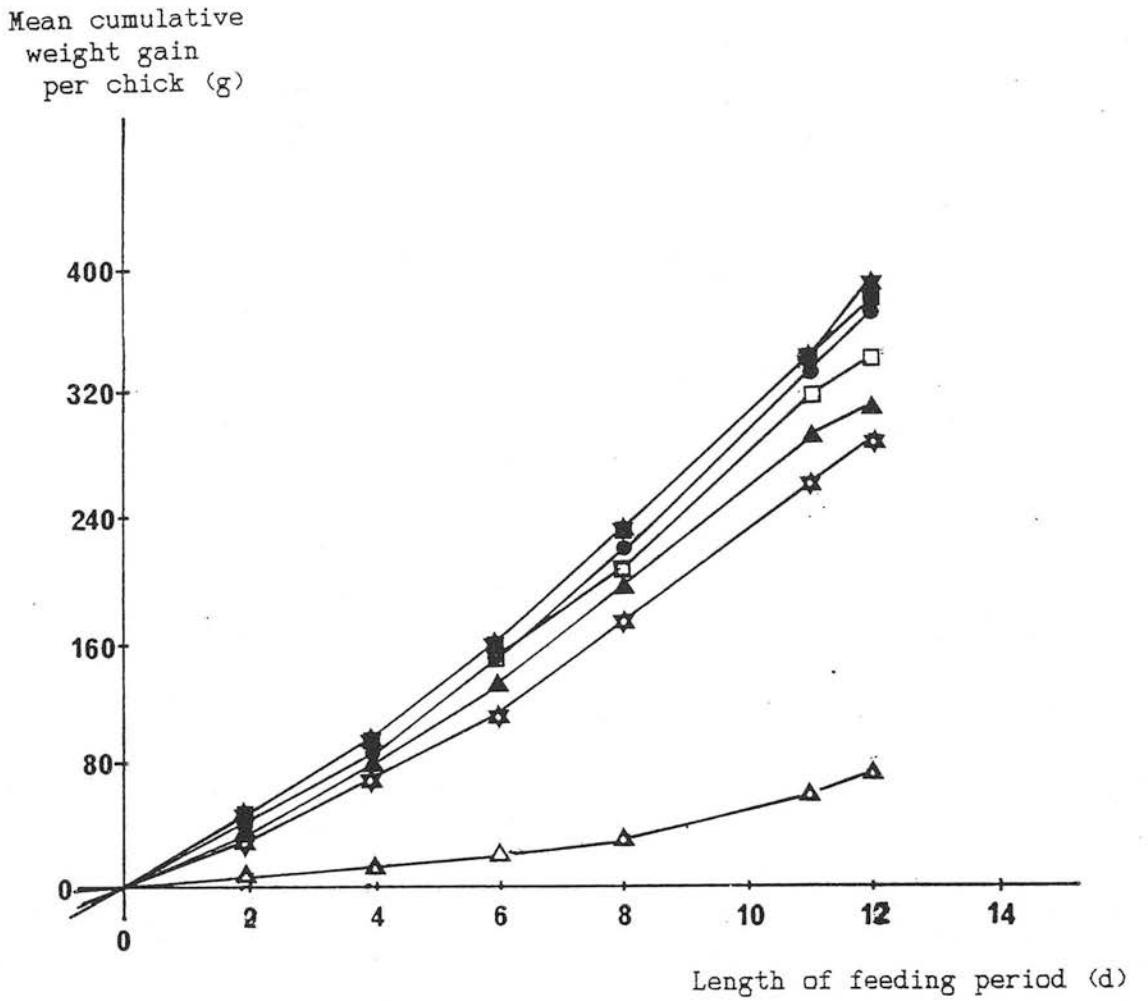


Fig. 27. Weight gain of chicks fed to determine the effect of increased incorporation of balanced and imbalanced proteins into the diet

●	I	Control diet having nitrogen content of 33.28g/kg
■	II	Control+casein equivalent of 16g nitrogen/kg diet
✱	III	Control+casein equivalent of 29g nitrogen/kg diet
▲	IV	Control+casein equivalent of 48g nitrogen/kg diet
□	V	Control+gelatin equivalent of 16g nitrogen/kg diet
✡	VI	Control+gelatin equivalent of 29g nitrogen/kg diet
△	VII	Control+gelatin equivalent of 48g nitrogen/kg diet

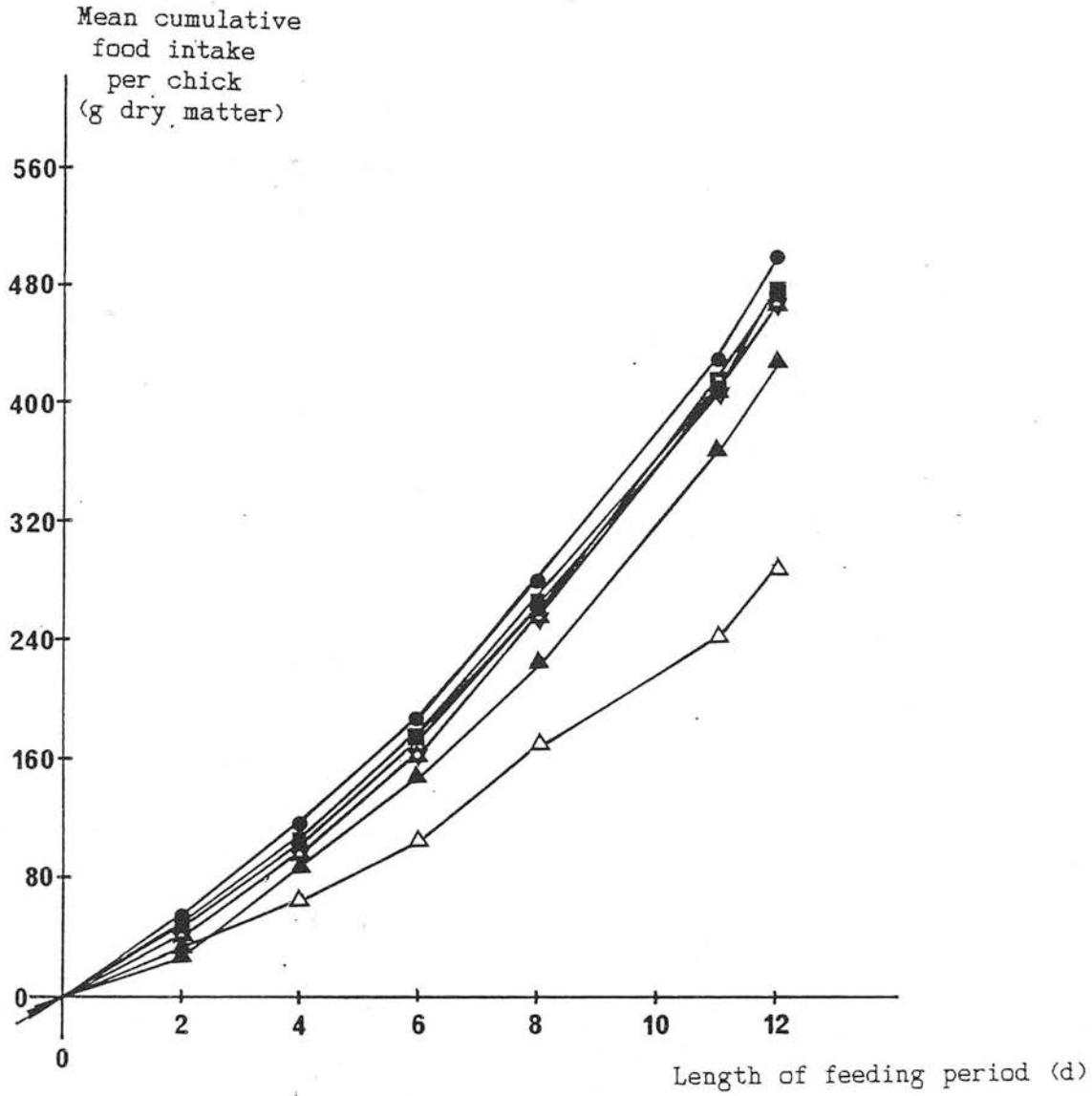


Fig. 28. Food intake of chicks fed to determine the effect of increased incorporation of balanced and imbalanced proteins into the diet

●	I	Control diet having nitrogen content of 33.28g/kg
■	II	Control+casein equivalent of 16g nitrogen/kg diet
✱	III	Control+casein equivalent of 29g nitrogen/kg diet
▲	IV	Control+casein equivalent of 48g nitrogen/kg diet
□	V	Control+gelatin equivalent of 16g nitrogen/kg diet
✡	VI	Control+gelatin equivalent of 29g nitrogen/kg diet
△	VII	Control+gelatin equivalent of 48g nitrogen/kg diet

incorporation, the difference being significant on the second ($P<0.05$), fourth ($P<0.01$), and sixth ($P<0.001$) days of the feeding period and thereafter ($P<0.01$). This fall in weight gain was accompanied by a reduction in food intake below that of birds fed the least incorporated amount of casein. This difference was initially highly significant ($P<0.001$) but attained lower levels of significance on the eighth ($P<0.01$), eleventh ($P<0.05$) and twelfth ($P<0.01$) days of the experiment. In comparison with birds fed the diet incorporating casein at a nitrogen level of 29g/kg the weight gain of chicks fed the maximum amount of casein was also reduced on days four ($P<0.01$), six ($P<0.001$), eight and eleven ($P<0.01$) and on the final day ($P<0.001$) of the experiment. Food intake was correspondingly reduced, the reduction being significant at a high level until the sixth day of feeding ($P<0.001$) and at lower levels after eight ($P<0.01$), eleven ($P<0.05$) and twelve ($P<0.01$) days of the experiment.

As with casein, addition of gelatin to the control diet at a level equivalent to an increase in dietary nitrogen content of 16g/kg had no significant effect on weight gain in comparison with birds fed the control diet alone. Food intake was very significantly depressed by day two ($P<0.001$) but attained lower levels of significance on day four ($P<0.01$) and day six ($P<0.05$), and was restored to control levels by day eight of the experimental period. Only on the eighth day of the experimental period did chicks fed this minimum quantity of gelatin show a significantly lower weight gain than those fed the diet incorporating the smallest amount of casein ($P<0.05$), there being no significant difference in food intake at any time. Similarly, the weight gain of birds receiving this gelatin-containing diet was below that of those fed the diet containing twice the minimum incorporated quantity of

casein, only on the eighth and twelfth days of feeding ($P<0.05$). The food intake of birds fed these two diets again was not significantly different.

The weight gain of chicks fed the diet containing the maximum incorporated quantity of casein was significantly below that of those receiving the minimum quantity of gelatin on the fourth and sixth days of the feeding period ($P<0.05$) but was not significantly different thereafter. The food intake of birds receiving the former diet was less than that of those fed the latter initially ($P<0.001$) and on the sixth day of feeding and thereafter ($P<0.01$).

Increasing dietary gelatin so as to raise the nitrogen content to 29g/kg above the concentration of the control diet caused a reduction in weight gain by the second day of feeding below that shown by chicks fed the control diet ($P<0.01$), the difference subsequently becoming very highly, significant ($P<0.001$). Food intake was initially reduced below that of the control diet ($P<0.001$), but this reduction had fallen in significance by the sixth day of feeding ($P<0.01$) and was not subsequently significant until the final day of the experiment ($P<0.05$). Chicks fed this diet containing twice the minimum incorporated amount of gelatin also had a lower weight gain than those fed the diet containing the least incorporated quantity of casein ($P<0.001$), the difference in food intake being significant on the second ($P<0.001$) and fourth ($P<0.01$) days of feeding but not significant thereafter.

Birds receiving the diet incorporating an amount of casein equivalent to a nitrogen content of 29g/kg again had a significantly greater gain in weight than those fed the diet containing gelatin sufficient to supply the same quantity of nitrogen, the difference being significant after two days of feeding ($P<0.01$) and for the remainder of

the experiment ($P<0.001$). The food intake of chicks fed the casein-containing diet was initially above that of those receiving the diet incorporating gelatin ($P<0.05$), but from the sixth day onwards was not significantly different. In comparison with birds fed the diet containing the maximum incorporated amount of casein, those consuming that containing twice the minimum amount of gelatin had a significantly lower weight gain on days four and six ($P<0.01$) and eleven ($P<0.05$) of the experimental period. The food intake of chicks receiving the latter diet was higher than that of those fed the former, the difference initially being very highly significant ($P<0.001$) but attaining lower levels of significance on the fourth and sixth ($P<0.05$), eighth ($P<0.01$), eleventh ($P<0.05$) and twelfth ($P<0.01$) days of feeding.

Chicks fed the diet containing twice the minimum incorporated amount of gelatin also had a lower weight gain than those receiving the minimum quantity of gelatin, the difference in weight gain being significant at the level of $P<0.05$ initially, $P<0.01$ after eight and twelve days of feeding and $P<0.001$ at all other times. Food intake was initially reduced relative to that of birds fed the minimum quantity of gelatin ($P<0.01$) but was restored on day six of feeding.

The incorporation of gelatin into the diet at a level sufficient to increase the dietary nitrogen content by 48g/kg resulted in immediate, highly significant reductions in both weight gain and food intake in comparison with the effects of all other diets fed ($P<0.001$).

c. Neurotransmitter concentrations

i. Dietary effects

During the HPLC analysis of the brain samples obtained from this investigation, the peak ratios calculated for DOPAC and HVA indicated that these eluted compounds were likely to be contaminated,

and data regarding their concentrations are therefore not presented. As shown in Table 25, the brain concentration of NE in birds fed the diet incorporating sufficient casein to raise the nitrogen content by 29g/kg was significantly lower than that of chicks fed the control diet ($P<0.01$), or the diet containing the minimum incorporated amount of casein ($P<0.05$). In comparison with birds fed the control diet, reduced brain concentrations of NE were also observed in chicks consuming diets in which gelatin increased the nitrogen content by 16g/kg ($P<0.01$) and 29g/kg ($P<0.05$).

Chicks receiving the diet incorporating the highest concentration of gelatin had a lower brain concentration of E than those fed that diet containing an amount of casein sufficient to raise the dietary nitrogen content by 16g/kg ($P<0.05$). Those fed the diet containing casein at its highest level of incorporation had significantly higher brain concentrations of E than birds receiving gelatin supplements raising the dietary nitrogen content by 16g/kg or 29g/kg ($P<0.05$), or 48g/kg ($P<0.01$). Brain DA concentrations of birds fed the greatest amount of gelatin were significantly above those of chicks receiving the diet containing twice the minimum incorporated quantity of casein ($P<0.05$).

Concentrations of 5HT in the brains of chicks receiving diets in which casein raised the nitrogen content by 29g/kg or gelatin raised it by 16g/kg or 48g/kg, were reduced significantly below those of birds fed the control diet ($P<0.05$). An increase in the brain concentration of 5HIAA of chicks receiving the greatest amount of dietary gelatin above that of those fed any of the casein-containing diets was also observed ($P<0.05$).

Table 25. Concentrations of neurotransmitters and metabolites in brains of chicks fed balanced and imbalanced proteins

Diet	Concentration of compound (ng/g fresh wt. brain tissue) NE	E	DA	5HT	5HIAA
I	361	35	298	755	110
II	339	38	304	704	90
III	283**	31	243	635**	90
IV	332	42	280	683	81
V	292**	30	248	626*	110
VI	303*	28	261	731	103
VII	323	25	319	641*	158
sem (18 d.f.)	17	4	25	37	20

Values significantly different from control values *P<0.05, **P<0.01, ***P<0.001

I	Control diet having nitrogen content of 33.28g/kg
II	Control+casein equivalent of 16g nitrogen/kg diet
III	Control+casein equivalent of 29g nitrogen/kg diet
IV	Control+casein equivalent of 48g nitrogen/kg diet
V	Control+gelatin equivalent of 16g nitrogen/kg diet
VI	Control+gelatin equivalent of 29g nitrogen/kg diet
VII	Control+gelatin equivalent of 48g nitrogen/kg diet

ii. Neurotransmitter concentrations and food intake

The brain concentration of DA in chicks receiving the experimental diets was significantly negatively correlated with their level of food intake ($r=0.434$, $P<0.05$). A positive correlation between the chick brain concentration of 5HT and the quantity of AME(N) consumed was also observed ($r=0.773$, $P<0.001$). No other significant correlation of the chick brain concentrations of NE, DA and 5HT with food intake or the quantities of protein or AME(N) ingested were apparent. The brain concentration of NE or DA was not significantly correlated with the level of intake of tyrosine+phenylalanine. Similarly, no significant correlation was observed between the chick brain concentration of 5HT and the amount of tryptophan consumed.

3.10. Experiment 9. The leucine-valine antagonism

a. Dietary nitrogen content

Determinations made on the diets employed in this experiment (Table 14) showed that the nitrogen content of the control diet was approximately 1.5g/kg below the nitrogen contents of the others, which differed only slightly from each other.

b. Growth and food intake (Figs. 29 and 30.)

After only two days of feeding the experimental diets, chicks consuming that incorporating the small supplement of valine showed a greater gain in weight than those fed the control diet alone ($P<0.05$). This difference in weight gain was significant at the level of $P<0.01$ om the fifth and twelfth days of feeding and at $P<0.05$ at all other times of measurement. The food intake of chicks consuming the diet supplemented only with valine was significantly greater than that of birds fed the control diet on the second and fifth ($P<0.01$), ninth and fourteenth ($P<0.05$) days of the feeding period, the difference in intake

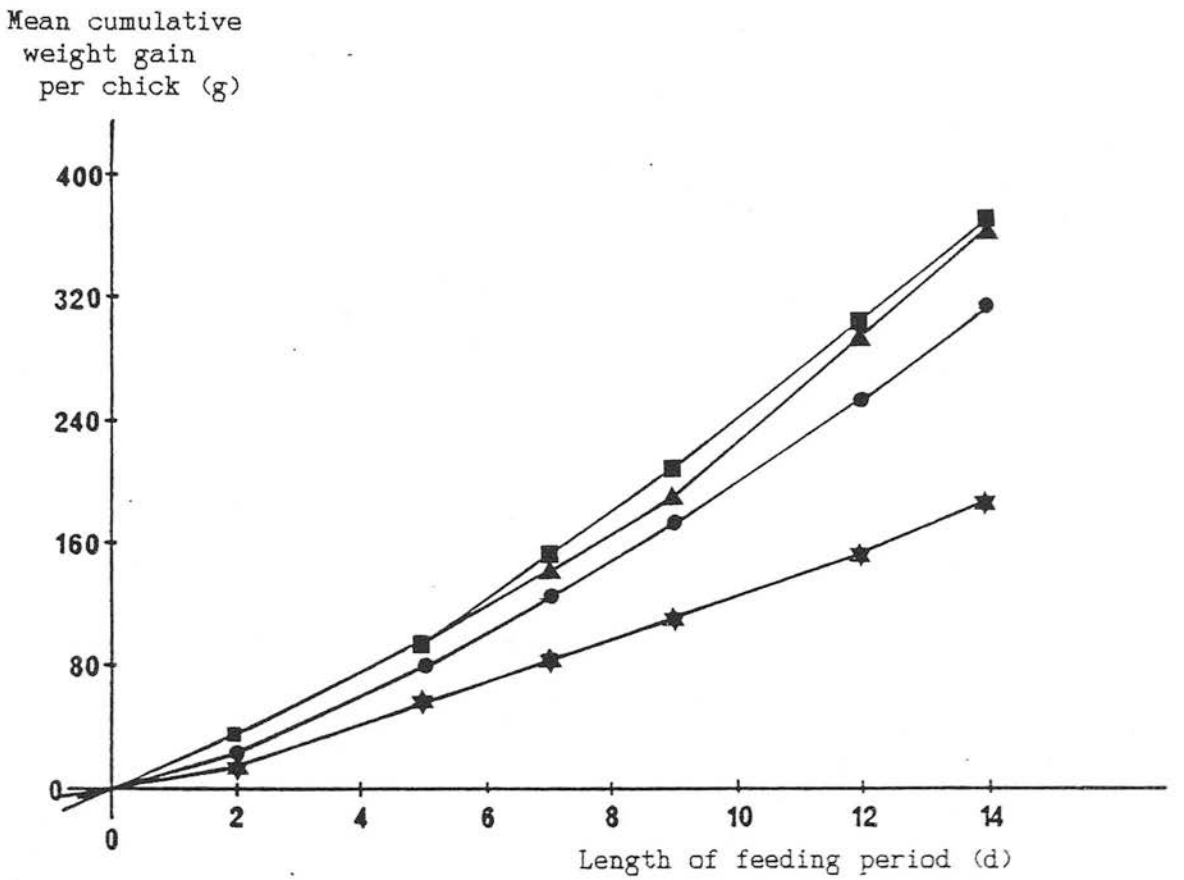


Fig. 29. Weight gain of chicks fed diets containing different concentrations of leucine and valine

- ● I Control diet having low valine content
- ■ II Control+valine (1.2g/kg diet)
- ★ III Control+leucine (40g/kg diet)
- ▲ IV Control+leucine (40g/kg diet)+valine (7g/kg diet)

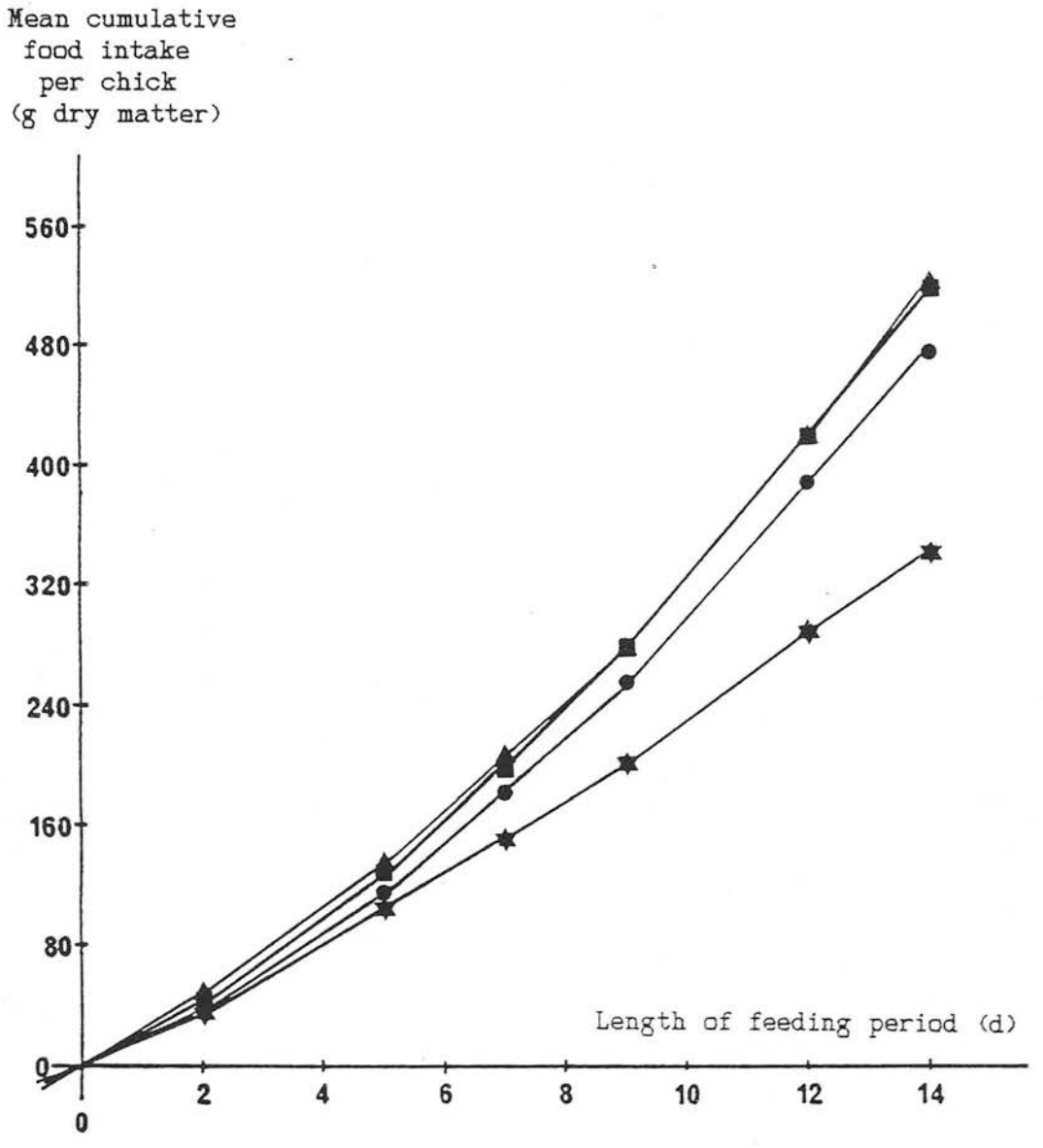


Fig. 30. Food intake of chicks fed diets containing different concentrations of leucine and valine

● I Control diet having low valine content
■ II Control+valine (1.2g/kg diet)
★ III Control+leucine (40g/kg diet)
▲ IV Control+leucine (40g/kg diet)+valine (7g/kg diet)

between these groups of chicks failing to reach significance on the seventh and twelfth days of feeding.

Chicks fed the diet containing excess dietary leucine had a significantly lower weight gain and food intake than those of birds receiving the control diet, this being apparent after only two days of feeding ($P<0.01$) and attaining a very high level of significance on days twelve and fourteen ($P<0.001$). The weight gain of birds fed the diet containing excess leucine alone was also significantly below that of those consuming the diet supplemented only with valine, the difference being significant on the second day of feeding ($P<0.01$) and thereafter ($P<0.001$). The food intake of chicks fed the former diet was constantly reduced below that of those receiving the former ($P<0.001$).

The weight gain of birds receiving the diet which was simultaneously supplemented with excess leucine and additional valine was initially significantly greater than that of those fed the control diet ($P<0.05$). The significance of this difference in growth was however lost by the seventh day of feeding and not regained until days twelve ($P<0.01$) and fourteen ($P<0.05$) of the experimental period. Chicks consuming the diet incorporating both excess leucine and supplemental valine also initially had a higher level of food intake than those receiving the control diet ($P<0.001$). The difference attained lower levels of significance after five ($P<0.01$) and seven ($P<0.05$) days of the feeding period and was not subsequently significant until the final day of the experiment ($P<0.05$).

In comparison with birds fed the diet supplemented only with valine, those receiving that incorporating both excess leucine and additional valine showed no significant difference in weight gain. The food intake of chicks consuming the latter diet was initially greater

than that of those fed the former ($P<0.01$), but from the seventh day of the feeding period onwards the levels of food intake of birds fed these diets were not significantly different. Both the weight gain and food intake of chicks consuming both excess leucine and supplemental valine were constantly significantly greater than those of birds receiving the diet incorporating excess leucine alone ($P<0.001$).

c. Neurotransmitter concentrations

i. Dietary effects

After five days of feeding the experimental diets, no significant differences in the brain concentrations of NE, E, DA, DOPAC and 5HT in chicks receiving the diets were observed (Table 26). Chicks fed the diet supplemented only with valine showed significantly higher brain concentrations of HVA and 5HIAA than those fed the basal diet or that containing both excess leucine and additional valine ($P<0.05$). The brain concentration of 5HIAA was also significantly greater in chicks consuming the diet supplemented with valine alone than in those fed the diet to which only excess leucine had been added ($P<0.01$).

In birds which had been receiving the diets for a period of nine days, the brain concentration of NE in those fed the diet supplemented only with valine was significantly increased above that of those fed either the control diet ($P<0.01$) or that incorporating both excess leucine and additional valine ($P<0.05$). Concentrations of E in the brains of birds fed additional valine alone or with excess leucine, were higher than in chicks consuming either of the other two diets ($P<0.05$). In birds consuming the diet supplemented solely with valine, the brain DA concentration was above that of chicks fed the control diet ($P<0.05$). Brain concentrations of DOPAC were not altered by the feeding of the different diets, while the concentration of HVA in the brains of chicks

Table 26. Brain concentrations of neurotransmitters and metabolites in chicks fed different concentrations of leucine and valine

Diet fed for period indicated	Concentration of compound (ng/g fresh brain tissue) NE	E	DA	DOPAC	HVA	5HT	5HIAA
5 Days							
Diet I	248	21	209	17	96	687	96
Diet II	231	16	183	26	139*	618	132*
Diet III	226	16	157	50	104	542	72
Diet IV	238	22	184	44	101	629	92
sem (6 d.f.)	35	5	32	12	11	66	8
9 Days							
Diet I	166	9	144	34	121	644	99
Diet II	303***	18*	242*	28	109	756	86
Diet III	226	7	191	22	76*	616	58*
Diet IV	206	18*	184	24	95	679	73
sem (6 d.f.)	25	2	27	9	12	41	8
14 Days							
Diet I	204	17	186	29	80	596	70
Diet II	237	20	230*	27	100	674	82*
Diet III	224	17	208	29	78	564	60
Diet IV	200	15	211	47	82	603	71
sem (6 d.f.)	21	2	11	6	9	41	3

Values significantly different from those of birds fed the control diet
*P<0.05, **P<0.01, ***P<0.001

I Control diet having low valine content
II Control+valine (1.2g/kg diet)
III Control+leucine (40g/kg diet)
IV Control+leucine (40g/kg diet)+valine (7g/kg diet)

receiving the diet containing excess leucine in the absence of additional valine was significantly reduced below that of birds fed the control diet ($P<0.05$).

The brain concentration of 5HT of chicks consuming the diet incorporating excess leucine alone was significantly less than that of birds receiving the diet supplemented only with valine ($P<0.05$). At the same time, 5HIAA was reduced to a concentration significantly below that of chicks fed either the control diet or that containing only additional valine ($P<0.05$).

After fourteen days, no significant difference in brain concentrations of NE, E, DOPAC, HVA and 5HT of chicks fed the various diets was observed. Significantly higher brain concentrations of DA and 5HIAA were found in chicks fed the diet supplemented with valine alone than in those fed the control diet ($P<0.05$). The concentration of 5HIAA in the brains of birds receiving the diet supplemented only with valine was also greater than that of chicks fed the diet incorporating excess leucine alone ($P<0.01$).

ii. Effects of age and length of feeding period

Chicks receiving the control diet had a significantly lower brain concentration of E after nine days of feeding than after only five ($P<0.05$). The brain concentrations of 5HIAA and HVA in birds fed the diet supplemented only with valine were also lower on the ninth day of the experimental period than on the fifth ($P<0.05$). At the end of the feeding period, the brain concentration of E in chicks receiving the diet containing excess leucine alone was significantly higher than in those which had received the diet for only nine days ($P<0.05$). Birds consuming the control diet had a lower brain concentration of HVA after fourteen days of feeding than after nine ($P<0.05$). Those fed the diet

supplemented only with valine had lower brain concentrations of 5HIAA ($P<0.01$) and HVA ($P<0.05$) than birds which had received the diet for only five days.

iii. Neurotransmitter concentrations and food intake

After nine days of feeding the experimental diets, the chick brain concentration of 5HT was positively correlated with intake of food as a whole and tryptophan in particular ($r=0.534$, $P<0.05$). It was also positively correlated with the amount of protein consumed ($r=0.557$, $P<0.05$). No other correlations of the chick brain concentrations of NE, DA or 5HT with food intake or the quantity of protein or AME(N) ingested was observed. At no time were the concentrations of NE or DA significantly correlated with the level of intake of tyrosine+phenylalanine or with the ratio of the dietary content of these amino acids to the sum of the concentrations of leucine+isoleucine+valine+tryptophan in the diet-these being the LNAA most likely to compete with the catecholamine precursors for uptake into the brain. Similarly, the chick brain concentration of 5HT was never significantly correlated with the amount of tryptophan consumed or the ratio of the dietary tryptophan content to the combined dietary concentrations of leucine+isoleucine+valine+tyrosine+phenylalanine. This latter group is of those LNAA most effective in competing with tryptophan for brain uptake.

3.11 Experiment 10. Effect of supplementary phenylalanine and tryptophan on the response of the chick to an increased dietary content of the branched-chain amino acids.

a. Dietary nitrogen and AME(N) content

Some variation in the nitrogen content of the diets fed in this investigation was apparent (Table 27), this tending to be higher in

diets containing supplements of the BCAA mixture with no further amino acid additions, than in the control diet. Diets containing additional tryptophan or phenylalanine had somewhat increased nitrogen contents. Those incorporating both of these amino acids however, tended to show a lower nitrogen content than diets containing the same concentration of the BCAA mixture but lacking these additions

The AME(N) contents of the control diet, that containing the lowest incorporated concentration of the BCAA mixture and that of the diet to which the highest concentration of the BCAA mixture and additional tryptophan at a concentration of 4g/kg had been added, were not significantly different from each other (Table 27). They were all however, below those of the other diets, the unsupplemented diet differing at a high level of significance ($P<0.001$). In a similar manner, the AME(N) content of the diet containing the lowest incorporated concentration of the BCAA mixture was below that of the diet containing the mixture at twice this concentration ($P<0.05$) and other diets ($P<0.01$). That diet incorporating the highest concentration of the BCAA mixture in combination with tryptophan had an AME(N) significantly below that of the diet containing the same concentration of the BCAA mixture in the absence of tryptophan ($P<0.01$) or the other diets ($P<0.05$).

b. Growth and food intake (Figs. 31 and 32.)

Throughout the experimental period, the weight gain and food intake of chicks fed the diet incorporating the lowest concentration of the BCAA mixture were not significantly different from those of birds receiving the control diet. However, chicks fed the diet incorporating twice the minimum amount of the BCAA mixture had a consistently reduced weight gain compared with birds consuming the

Table 27. Nitrogen and AME(N) contents of diets fed during determination of the effects of supplementary phenylalanine and tryptophan on the response of the chick to an increased dietary content of the branched-chain amino acids.

	DIET FED							
	I	II	III	IV	V	VI	VII	VIII
NITROGEN CONTENT (g/kg dry matter)	35.60	38.40	36.80	34.80	38.00	39.20	38.40	36.40
AME(N) (MJ/kg dry matter)	14.21	14.64	15.40***	15.52***	15.70***	14.75	15.57***	15.67***

sem (21 d.f.)=0.22

Values significantly different from that of control diet ***P<0.001

I	Control diet
II	Control+leucine (20g/kg diet)+isoleucine (11.6g/kg diet)+valine (13.3g/kg diet)
III	Control+leucine (40g/kg)+isoleucine (23.1g/kg) +valine (26.7g/kg)
IV	Control+leucine (40g/kg)+isoleucine (23.1g/kg), +valine (26.7g/kg)+tryptophan (4g/kg)+phenylalanine(8g/kg)
V	Control+leucine (60g/kg)+isoleucine (11.6g/kg) +valine (13.3g/kg)
VI	Control+leucine (60g/kg)+isoleucine (11.6g/kg), +valine (13.3g/kg)+tryptophan (4g/kg)
VII	Control+leucine (60g/kg)+isoleucine (11.6g/kg), valine (13.3g/kg)+phenylalanine (8g/kg)
VIII	Control+leucine (60g/kg)+isoleucine (11.6g/kg), +valine (13.3g/kg)+tryptophan (4g/kg)+phenylalanine(8g/kg)

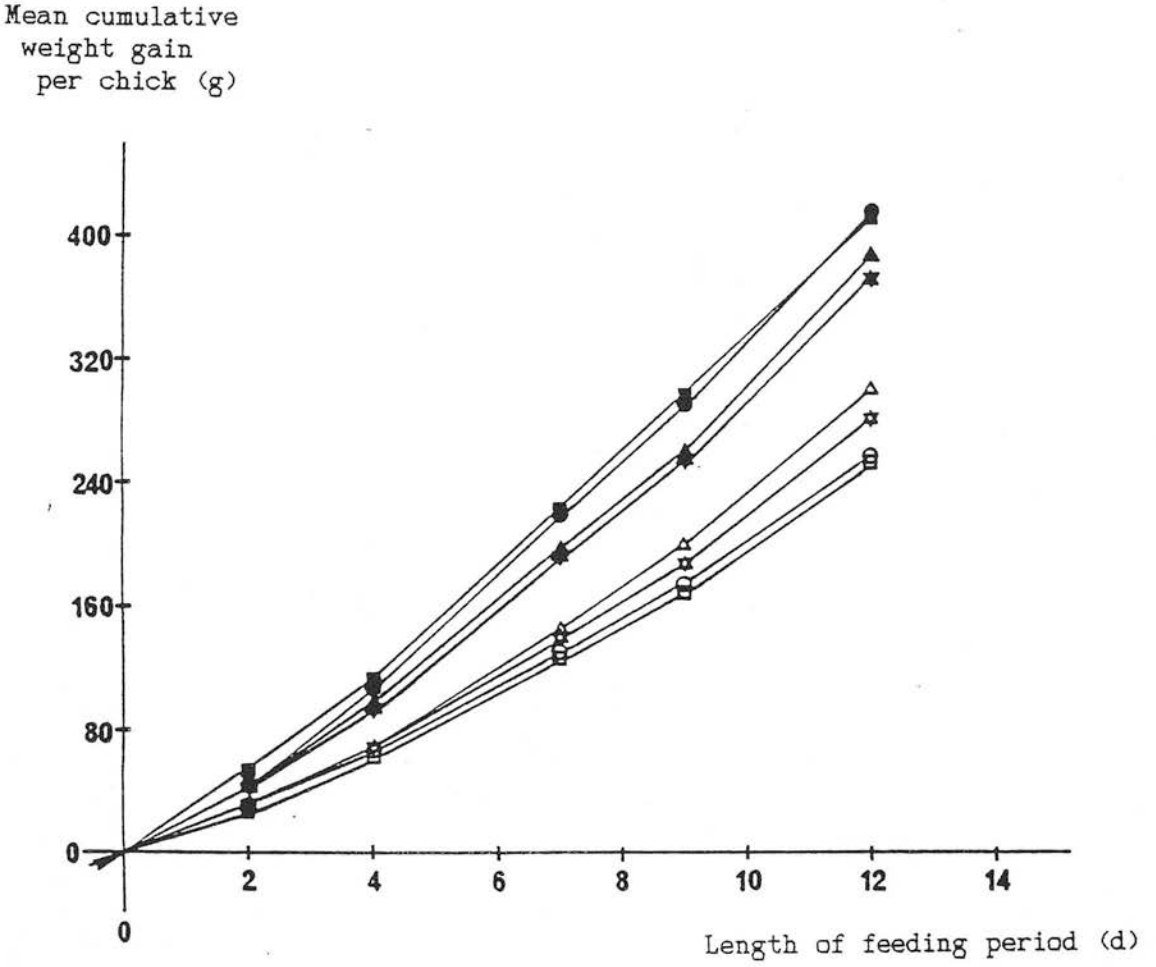


Fig. 31. Weight gain of chicks fed increasing amounts of a mixture of the branched-chain amino acids, alone or with supplemental phenylalanine and/or tryptophan

●	I	Control diet
■	II	Control+leucine (20g/kg diet)+isoleucine (11.6g/kg diet)+valine (13.3g/kg diet)
✱	III	Control+leucine (40g/kg)+isoleucine (23.1g/kg) +valine (26.7g/kg)
▲	IV	Control+leucine (40g/kg)+isoleucine (23.1g/kg), +valine (26.7g/kg)+tryptophan (4g/kg)+phenylalanine(8g/kg)
○	V	Control+leucine (60g/kg)+isoleucine (11.6g/kg) +valine (13.3g/kg)
□	VI	Control+leucine (60g/kg)+isoleucine (11.6g/kg), +valine (13.3g/kg)+tryptophan (4g/kg)
✡	VII	Control+leucine (60g/kg)+isoleucine (11.6g/kg), valine (13.3g/kg)+phenylalanine (8g/kg)
△	VIII	Control+leucine (60g/kg)+isoleucine (11.6g/kg), +valine (13.3g/kg)+tryptophan (4g/kg)+phenylalanine(8g/kg)

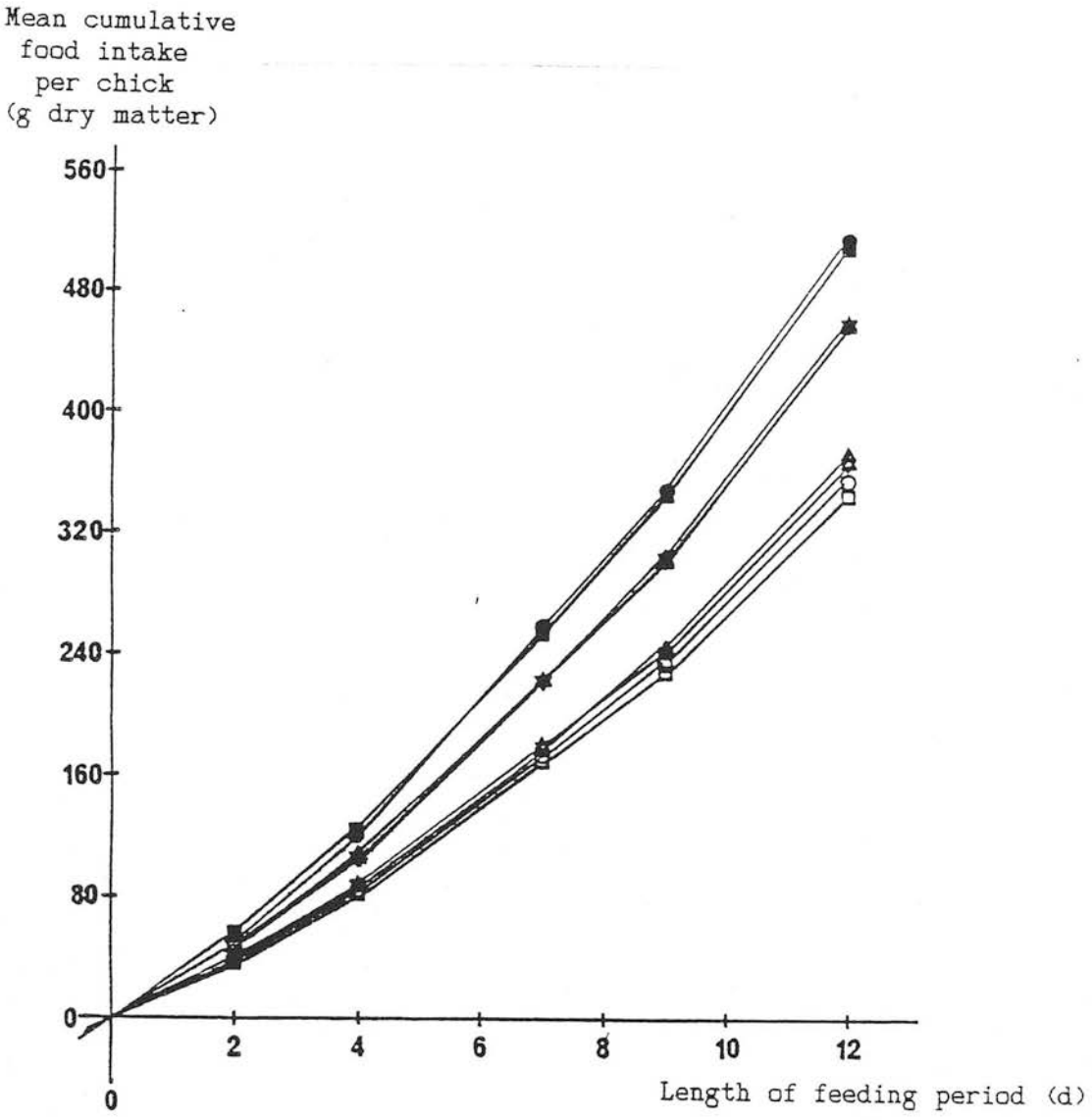


Fig. 32 Food intake of chicks fed increasing amounts of a mixture of the branched-chain amino acids, alone or with supplemental phenylalanine and/or tryptophan

● I	Control diet
■ II	Control+leucine (20g/kg diet)+isoleucine (11.6g/kg diet)+valine (13.3g/kg diet)
✱ III	Control+leucine (40g/kg)+isoleucine (23.1g/kg) +valine (26.7g/kg)
▲ IV	Control+leucine (40g/kg)+isoleucine (23.1g/kg), +valine (26.7g/kg)+tryptophan (4g/kg)+phenylalanine(8g/kg)
○ V	Control+leucine (60g/kg)+isoleucine (11.6g/kg) +valine (13.3g/kg)
□ VI	Control+leucine (60g/kg)+isoleucine (11.6g/kg), +valine (13.3g/kg)+tryptophan (4g/kg)
✡ VII	Control+leucine (60g/kg)+isoleucine (11.6g/kg), valine (13.3g/kg)+phenylalanine (8g/kg)
△ VIII	Control+leucine (60g/kg)+isoleucine (11.6g/kg), +valine (13.3g/kg)+tryptophan (4g/kg)+phenylalanine(8g/kg)

control diet ($P<0.01$). The food intake of chicks receiving the former diet was also below that of those fed the control diet, the difference being significant on the fourth day of the feeding period ($P<0.01$) and thereafter ($P<0.001$). Birds fed the diet containing twice the minimum incorporated amount of the BCAA mixture also had a level of weight gain which was less than that of those consuming half this amount, the difference being significant after four ($P<0.001$), seven ($P<0.01$), nine and twelve ($P<0.001$) days of the experiment. Food intake was similarly reduced after two days of feeding ($P<0.05$) and on the fourth day of the feeding period and thereafter ($P<0.001$).

The addition of phenylalanine and tryptophan to the diet incorporating twice the minimum concentration of the BCAA mixture resulted in no significant improvement in weight gain, this being significantly lower than that of those fed the control diet on days four and twelve of the experimental period ($P<0.05$) and at all times of measurement between these points ($P<0.01$). Food intake of birds fed such a supplemented diet was also reduced below that of those fed the control diet, the difference being significant on the fourth day of feeding ($P<0.01$) and thereafter ($P<0.001$). In comparison with those fed the diet containing the minimum supplement of the BCAA mixture alone, the weight gain of chicks receiving twice this amount of the mixture and both phenylalanine and tryptophan was significantly reduced on the fourth and seventh ($P<0.01$), ninth ($P<0.001$) and twelfth ($P<0.05$) days of the experimental period. The food intake of chicks receiving the latter diet was also less than that of those fed the former, the difference being significant after two days of feeding ($P<0.05$) and at all subsequent times of measurement ($P<0.001$). Neither the growth nor food intake of birds consuming the diet incorporating twice the minimum concentration

of the BCAA mixture together with supplements of phenylalanine and tryptophan was significantly different from that of those fed the diet containing the same quantity of the BCAA mixture alone.

The weight gain and food intake of birds fed the diet incorporating the maximum concentration of the BCAA mixture alone were reduced below those of chicks receiving the control diet, the differences being observable immediately ($P<0.01$) and very highly significant from the fourth day of feeding onwards ($P<0.001$). In comparison with those fed the diet incorporating the minimum quantity of the BCAA mixture, both the weight gain and food intake of birds fed the diet containing the maximum amount of this mixture were very significantly reduced from the second day of the feeding period onwards ($P<0.001$). The diets containing twice the minimum incorporated quantity of the BCAA mixture alone or with supplemental phenylalanine and tryptophan supported greater levels of weight gain than did the diet containing the maximum amount, the differences being significant after two days of feeding ($P<0.01$) and thereafter ($P<0.001$). On the second day of the experimental period, the food intake of birds receiving the maximum amount of the BCAA mixture was less than that of birds fed the diets containing twice the minimum incorporated amount alone ($P<0.05$) or with both phenylalanine and tryptophan ($P<0.01$). These differences in food intake were subsequently very highly significant ($P<0.001$).

Chicks receiving the diet having maximum BCAA supplementation and containing additional tryptophan continued to show a significantly lower weight gain and food intake than those fed the control diet or that incorporating the minimum quantity of the BCAA mixture ($P<0.001$). Birds fed the former diet also had a weight gain and food intake which were below those of chicks fed diets containing twice

the minimum incorporated amount of the BCAA mixture alone or with additional phenylalanine and tryptophan. The differences were significant on the second day of feeding ($P<0.01$) and thereafter. In comparison with birds fed the diet containing the maximum amount of the BCAA mixture alone, the weight gain and food intake of those receiving a concomitant supplement of tryptophan were not significantly different.

The diet incorporating the maximum amount of the BCAA mixture together with a supplement of phenylalanine rather than tryptophan supported a lower weight gain and food intake than the control diet, the difference being significant on day two of the feeding period ($P<0.01$) and thereafter ($P<0.001$). Both the weight gain and food intake of birds fed the former diet were constantly reduced below that of those receiving the diet containing the minimum amount of the BCAA mixture ($P<0.001$). In comparison with birds receiving the diets containing twice the minimum incorporated amount of the BCAA mixture alone or with phenylalanine and tryptophan, the weight gain ($P<0.01$) and food intake ($P<0.05$) of chicks fed the phenylalanine-supplemented diet containing the maximum amount of the BCAA mixture was significantly reduced after two days of feeding. These reductions subsequently became very highly significant ($P<0.001$). Neither the weight gain nor food intake supported by the diet containing the maximum amount of the BCAA mixture and supplemental phenylalanine was significantly different from that supported by the diet incorporating the same amount of the BCAA mixture alone, the somewhat greater weight gain of those fed the former diet just failing to reach significance on the final day of the experiment. No difference in food intake was apparent between chicks fed the maximum amount of the BCAA mixture with a supplement of tryptophan and those fed the same amount of the mixture with a supplement of

phenylalanine. However, the weight gain of chicks fed the latter diet was significantly greater than that of those receiving the former on the final day of the feeding period ($P<0.05$)

On the second day of the experimental period, the weight gain and food intake of chicks fed the diet containing the maximum amount of the BCAA mixture together with supplements of both phenylalanine and tryptophan was significantly below those of birds fed the control diet ($P<0.01$) or that incorporating the minimum amount of the BCAA mixture ($P<0.001$). At subsequent times of measurement, these observed differences in weight gain and food intake were very highly significant ($P<0.001$). In comparison with birds receiving the diets containing twice the minimum incorporated amount of the BCAA mixture alone or with supplemental phenylalanine and tryptophan, the weight gain of those fed the diet having the maximum BCAA content and supplemented with phenylalanine and tryptophan was reduced on the second day of the experiment ($P<0.01$) and thereafter ($P<0.001$). After two days of feeding, the food intake of birds fed the latter diet was also less than that of those receiving the diets containing twice the minimum incorporated amount of the BCAA mixture alone ($P<0.05$) or with phenylalanine and tryptophan ($P<0.01$). These differences in food intake were subsequently very highly significant ($P<0.001$).

A significant increase in the weight gain of birds fed the diet containing the maximum amount of the BCAA mixture and supplements of both phenylalanine and tryptophan above that of those fed the same quantity of the BCAA mixture alone, was apparent after nine ($P<0.05$) and twelve ($P<0.01$) days of the experiment. No significant difference in the food intake of birds fed these two diets was observed. On the seventh day of feeding, chicks fed the diet containing both tryptophan and

phenylalanine supplements in addition to the maximum amount of the BCAA mixture had a weight gain which was significantly greater than that of those fed the similar diet which lacked phenylalanine supplementation ($P<0.05$) The level of significance of this difference in weight gain had increased by the ninth day of feeding ($P<0.01$) and was further improved on the final day of the experiment ($P<0.001$). Only on the ninth day of the feeding period was the increased weight gain accompanied by a significantly increased level of food intake ($P<0.05$). At no time was the weight gain and food intake of chicks fed the diet containing the maximum amount of the BCAA mixture and supplements of both tryptophan and phenylalanine significantly different from that of those receiving the diet incorporating the same amount of the BCAA mixture but supplemented only with phenylalanine.

c. Neurotransmitter concentrations

i. Dietary effects

Epinephrine was undetectable in the brains of chicks fed the diets of this investigation. It is apparent from Table 28 that chicks fed the diet incorporating the minimum amount of the BCAA mixture had a lower brain concentration of DA and a higher concentration of the DA metabolite HVA than those receiving the control diet ($P<0.05$). The brain concentration of HVA in chicks consuming the former diet was also significantly greater than that of birds fed the diet containing twice the quantity of the BCAA mixture alone ($P<0.01$). Chicks receiving this latter diet had a significantly lower brain concentration of 5HIAA than those fed the control diet ($P<0.05$).

The concentrations of DA and 5HIAA in the brains of chicks receiving the diet incorporating the BCAA mixture at twice its minimum concentration and supplements of both phenylalanine and tryptophan, were

Table 28. Brain concentrations of neurotransmitters and metabolites in chicks fed increasing amounts of a mixture of the branched-chain amino acids, alone or with supplemental phenylalanine and/or tryptophan

Diet	Concentration of compound (ng/g fresh wt. brain tissue) NE	DA	DOPAC	HVA	5HT	5HIAA
I	296	246	68	90	572	169
II	273	191*	132	140*	547	152
III	251	202	78	77	467	120*
IV	262	180*	83	94	463	117*
V	186**	150**	101	57	357***	73***
VI	178***	150**	84	77	445*	116**
VII	274	161**	135	88	313***	79***
VIII	269	221	88	71	470	123*
sem (21 d.f.)	22	18	31	15	36	15

Values significantly different from control values *P<0.05, **P<0.01, ***P<0.001

I Control diet
II Control+leucine (20g/kg diet)+isoleucine (11.6g/kg diet) +valine (13.3g/kg diet)
III Control+leucine (40g/kg)+isoleucine (23.1g/kg) +valine (26.7g/kg)
IV Control+leucine (40g/kg)+isoleucine (23.1g/kg), +valine (26.7g/kg)+tryptophan (4g/kg)+phenylalanine(8g/kg)
V Control+leucine (60g/kg)+isoleucine (11.6g/kg) +valine (13.3g/kg)
VI Control+leucine (60g/kg)+isoleucine (11.6g/kg), +valine (13.3g/kg)+tryptophan (4g/kg)
VII Control+leucine (60g/kg)+isoleucine (11.6g/kg), +valine (13.3g/kg)+phenylalanine (8g/kg)
VIII Control+leucine (60g/kg)+isoleucine (11.6g/kg), +valine (13.3g/kg)+tryptophan (4g/kg)+phenylalanine(8g/kg)

below those of birds fed the control diet ($P<0.05$). In comparison with chicks fed the diet containing the minimum quantity of the BCAA mixture, those consuming the diet which contained twice this amount and was also supplemented with phenylalanine and tryptophan had a reduced brain concentration of HVA ($P<0.05$).

Birds fed the diet incorporating the highest level of the BCAA mixture alone had brain concentrations of NE and DA which were significantly below those of chicks receiving the control diet ($P<0.01$). The concentration of NE in the brains of chicks receiving the diet containing the maximum incorporated amount of the BCAA mixture was also less than that of birds fed the diets containing the minimum amount of this mixture ($P<0.01$) or twice the minimum amount alone or with phenylalanine and tryptophan ($P<0.05$). In comparison with birds fed the diet containing the minimum amount of the BCAA mixture alone, those receiving that incorporating the maximum quantity of this mixture had a significantly lower brain concentration of HVA ($P<0.001$).

The brain concentrations of 5HT and 5HIAA in chicks consuming the diet containing the maximum amount of the BCAA mixture alone were reduced in comparison with chicks fed the control diet ($P<0.001$) or that containing the minimum amount of the BCAA mixture ($P<0.01$). The concentration of 5HIAA in the brains of birds fed the diet containing twice the minimum incorporated amount of the BCAA mixture alone was also greater than that of chicks receiving the diet incorporating the maximum quantity of the mixture in the absence of tryptophan or phenylalanine supplements ($P<0.05$), the higher 5HT concentration of those fed the former diet just failing to reach significance.

Chicks receiving the diet containing the maximum amount of the BCAA mixture and supplemental tryptophan had significantly lower brain concentrations of NE ($P<0.001$), DA ($P<0.01$), 5HT ($P<0.05$) and 5HIAA ($P<0.01$) than those fed the control diet. The brain concentration of NE in birds fed the former diet was also below that of chicks receiving the diets containing the minimum incorporated quantity of the BCAA mixture ($P<0.01$), or twice this amount alone or with additional tryptophan and phenylalanine ($P<0.05$). Chicks receiving the tryptophan-supplemented diet containing the maximum quantity of the BCAA mixture also had a significantly lower brain concentration of HVA than those fed the diet incorporating the minimum amount of this mixture ($P<0.01$).

Supplementation of the diet containing the maximum quantity of the BCAA mixture with phenylalanine instead of tryptophan, restored the brain NE concentration of chicks fed this diet to that of birds fed the control diet. The brain concentration of NE in chicks receiving the phenylalanine-supplemented diet containing the maximum amount of the BCAA mixture was thus significantly higher than that of those fed the diets containing the maximum amount of the BCAA mixture alone or with additional tryptophan ($P<0.01$). Brain concentrations of HVA ($P<0.05$), 5HT ($P<0.001$) and 5HIAA ($P<0.01$) were lower in chicks consuming the phenylalanine-supplemented diet containing the greatest amount of the BCAA mixture than in those receiving the diet incorporating the minimum quantity of the mixture. Chicks fed the former diet also had a significantly lower brain concentration of 5HT than those receiving diets containing twice the minimum incorporated quantity of the BCAA mixture alone or with supplemental tryptophan and phenylalanine ($P<0.01$), or the diet containing the maximum amount of the BCAA mixture and a tryptophan supplement ($P<0.05$).

Birds fed the diet containing the maximum amount of the BCAA mixture and supplements of both phenylalanine and tryptophan had brain concentrations of NE, DA and 5HT which were not significantly different from those of birds fed the control diet. The concentration of DA in the brains of birds receiving the former diet was also significantly greater than that of chicks fed diets containing the maximum amount of the BCAA mixture alone or with a supplement of either tryptophan or phenylalanine ($P<0.05$). Chicks fed the diet incorporating the minimum quantity of the BCAA mixture again had a higher brain concentration of HVA than those consuming the diet containing the maximum amount of the BCAA mixture and supplements of tryptophan and phenylalanine ($P<0.01$). The brain concentration of 5HT in chicks fed the latter diet was higher than that of those receiving the diets containing the maximum amount of the BCAA mixture alone ($P<0.05$) or with supplements of tryptophan ($P<0.05$) or phenylalanine ($P<0.01$). The concentration of 5HIAA in the brains of chicks fed the diet containing the greatest quantity of the BCAA mixture and supplemented with tryptophan and phenylalanine was significantly greater than that of birds receiving the same amount of the BCAA mixture alone ($P<0.05$), but less than that of those consuming the control diet ($P<0.05$).

ii. Neurotransmitter concentrations and food intake

The brain concentration of NE in chicks fed the diets of this investigation was positively correlated with the level of food intake ($r=0.473$, $P<0.01$) and the quantity of protein ($r=0.412$, $P<0.05$) or AME(N) ($r=0.468$, $P<0.01$) consumed. It was also positively correlated with the combined amount of tyrosine+phenylalanine ingested ($r=0.551$, $P<0.001$). The brain concentration of DA was similarly positively correlated with food intake ($r=0.443$, $P<0.05$), protein intake ($r=0.374$,

P<0.05) and AME(N) intake (r=0.404, P<0.05). However, no correlation of the brain DA concentration with the quantity of tyrosine+phenylalanine consumed was observed. A positive correlation was apparent between the brain concentrations of both NE (r=0.505) and DA (r=0.501) and the ratio of the dietary content of tyrosine+phenylalanine to the sum of the dietary concentrations of leucine+isoleucine+valine+tryptophan. Both these correlations were significant at the level of P<0.05.

The concentration of 5HT in the brains of chicks receiving the experimental diets was also positively correlated with the total food intake (r=0.57, P<0.001) and the amounts of protein (r=0.545, P<0.01) and AME(N) ingested (r=0.491, P<0.01). Brain 5HT concentrations were not correlated with the quantity of tryptophan consumed, but were positively correlated with the ratio of the dietary content of tryptophan to the combined concentrations of leucine+isoleucine+valine+tyrosine+phenylalanine in the diet (r=0.525, P<0.05).

3.12 Experiment 11. Excessive dietary content of branched-chain amino acids and increased supplementation with phenylalanine and tryptophan

a. Dietary nitrogen and AME(N) content

The nitrogen content of the experimental diets (Table 29) increased with increasing supplementation of the diet with amino acids. The AME(N) of the unsupplemented control diet was significantly below those of the other three diets (P<0.001), which were in turn not significantly different from each other (Table 29.).

b. Growth and food intake (Figs. 33 and 34.)

Chicks receiving the control diet showed very similar patterns of growth and intake to those fed the identical diet in the previous experiment. Birds fed the diet incorporating the BCAA mixture showed an immediate depression in weight gain (P<0.01) and food intake

Table 29. Nitrogen and AME(N) content of diets containing excessive amounts of branched-chain amino acids in the presence and absence of increased concentrations of tryptophan and phenylalanine

	DIET FED			
	I	II	III	IV
NITROGEN CONTENT (g/kg dry matter)	34.84	49.29	49.79	51.41
AME(N) (MJ/kg dry matter)	13.61	15.51***	15.61***	15.57***

sem (6 d.f.)=0.20

Values significantly different from that of control diet, ***P<0.001

I Control diet
II Control diet+leucine (60g/kg diet)+isoleucine (34.7g/kg diet)+valine (40g/kg diet)
III Control diet+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg)+phenylalanine (12g/kg)
IV Control diet+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg)+phenylalanine (12g/kg)+tryptophan (8g/kg)

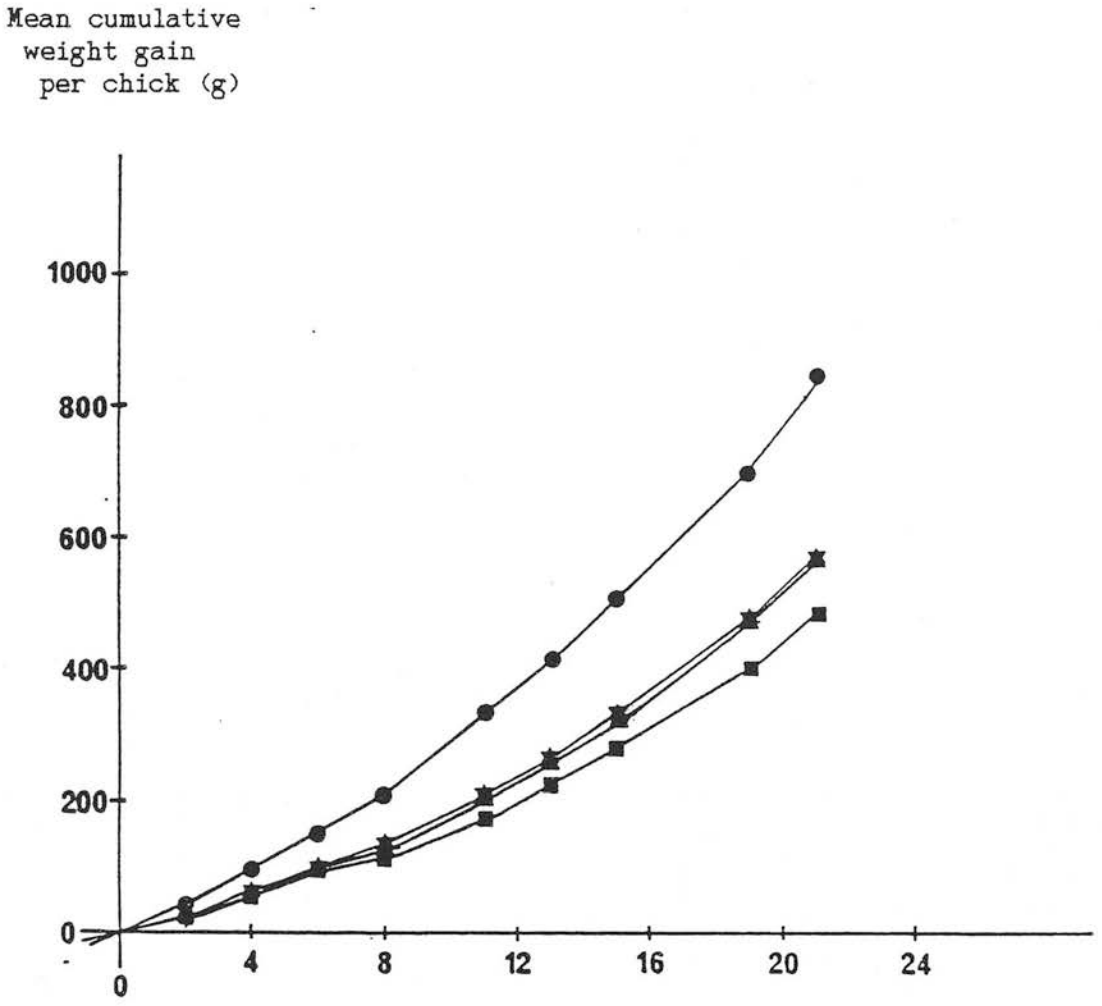


Fig. 33. Weight gain of chicks fed an excessive amount of a branched-chain amino acid mixture alone and with increased concentrations of tryptophan and phenylalanine

- ● I Control diet
- ■ II Control+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg)
- ★ III Control+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg) +phenylalanine (12g/kg)
- ▲ IV Control+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg) +phenylalanine (12g/kg)+tryptophan (8g/kg)

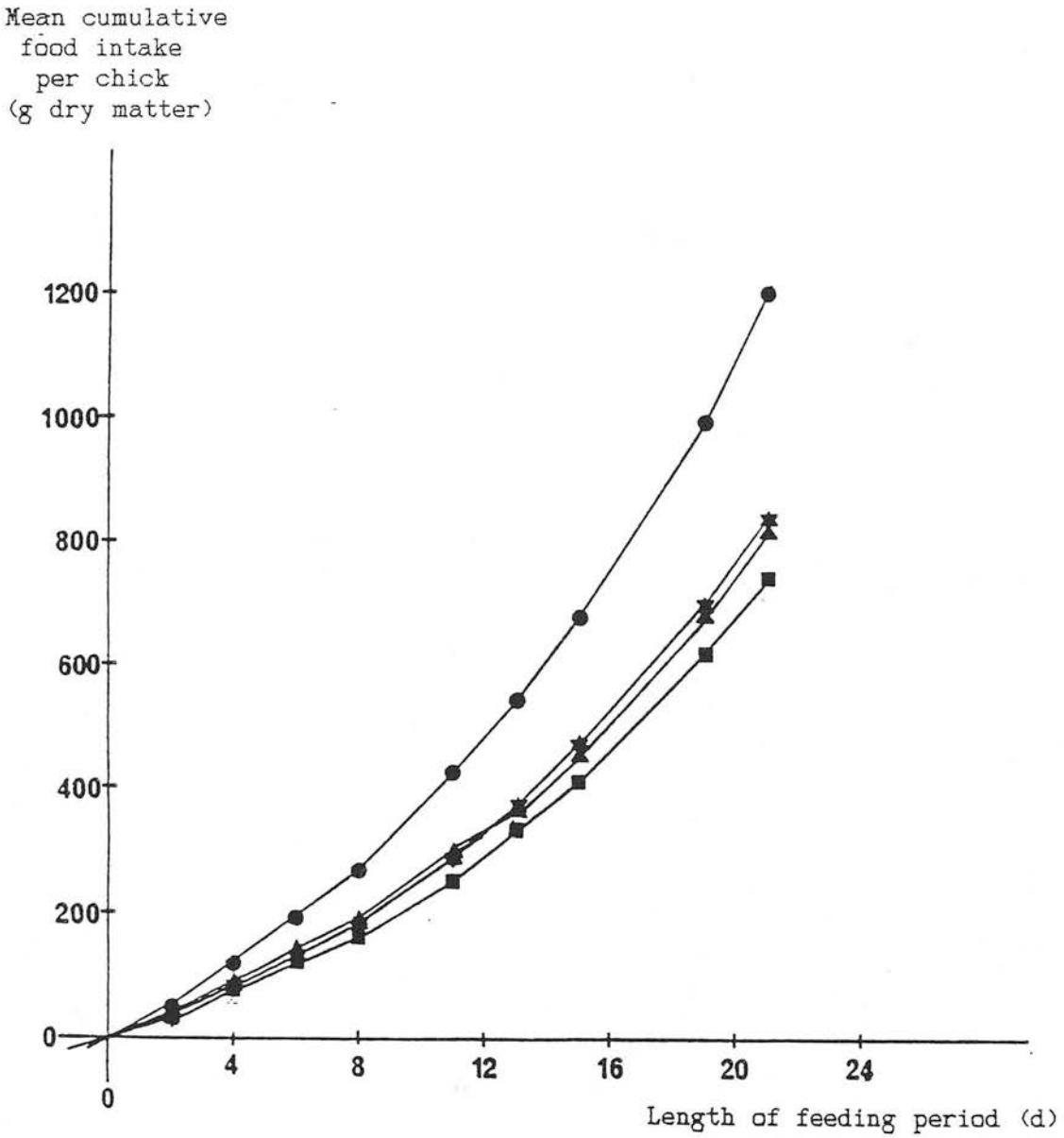


Fig. 34. Food intake of chicks fed an excessive amount of a branched-chain amino acid mixture alone and with increased concentrations of tryptophan and phenylalanine

- ● I Control diet
- ■ II Control+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg)
- ★ III Control+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg) +phenylalanine (12g/kg)
- ▲ IV Control+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg) +phenylalanine (12g/kg)+tryptophan (8g/kg)

($P<0.05$) in comparison with chicks fed the control diet. The reduction in weight gain was significant on the fourth ($P<0.001$) and sixth ($P<0.01$) days of feeding, and very highly significant thereafter ($P<0.001$). Food intake was similarly depressed below that of birds fed the control diet, the reduction attaining significance at the level of $P<0.01$ on the sixth day of feeding, $P<0.05$ at the end of the experiment and being very highly significant at all other times of measurement ($P<0.001$).

The weight gain of birds consuming the diet incorporating both the BCAA mixture and additional phenylalanine was still significantly lower than that of those fed the control diet after two ($P<0.01$), four ($P<0.001$), six and eight ($P<0.01$) days of the experimental period and thereafter ($P<0.001$). The food intake of chicks fed the former diet was also below that of those fed the latter, the difference in food intake attaining significance on days two ($P<0.05$), six ($P<0.01$) and twenty-one ($P<0.05$) of feeding and at all other times of measurement ($P<0.001$). Birds receiving the diet containing both the BCAA mixture and the phenylalanine supplement tended to have a greater weight gain and food intake than those fed the diet containing the BCAA mixture alone. However, this increase in weight gain attained significance only on the eleventh and fifteenth days of feeding ($P<0.05$), while a significant increase in food intake was observable only on the fifteenth day of the experimental period ($P<0.05$).

Chicks fed the diet incorporating the BCAA mixture and supplements of both tryptophan and phenylalanine again had a significantly lower weight gain than those fed the control diet, this being observable after two days of feeding ($P<0.05$). The reduction in weight gain attained significance at the level of $P<0.01$ on the fourth

day of the experiment and from the eleventh day onwards was very highly significant ($P<0.001$). The food intake of the chicks fed the diet containing the BCAA mixture and both phenylalanine and tryptophan supplements was also reduced below that of birds fed the control diet on days two ($P<0.05$), six ($P<0.01$) and twenty-one ($P<0.05$) of feeding and at all other times of measurement ($P<0.001$). This depression in food intake below that of birds fed the control diet was similar to that seen for chicks fed the other diets containing the BCAA mixture.

No significant difference in weight gain was apparent between chicks fed the diet incorporating the BCAA mixture with supplements of phenylalanine and tryptophan and those consuming the diets containing the BCAA mixture alone or with only supplemental phenylalanine. The only significant difference in the food intake of chicks fed these three diets was observed on the eleventh day of feeding, when that of birds receiving the diet containing the BCAA mixture with supplements of both phenylalanine and tryptophan was greater than that of those fed the diet containing the BCAA mixture alone ($P<0.05$).

c. Neurotransmitter concentrations

i. Dietary effects

Determination of the brain concentrations of E in chicks receiving the different diets of this investigation was not carried out, due to a suspected contamination of the E peak as eluted from the HPLC system. After six days of feeding, no significant differences in the brain concentrations of NE, DA, DOPAC or HVA in birds consuming the different diets were observed (Table 30). The concentration of 5HT in the brains of chicks receiving either the control diet or that containing the BCAA mixture with additional tryptophan and

Table 30. Brain concentrations of neurotransmitters and metabolites in chicks fed an excessive amount of a branched-chain amino acid mixture alone and with increased concentrations of tryptophan and phenylalanine

Diet fed for period indicated	Concentration of compound (ng/g fresh brain tissue) NE	DA	DOPAC	HVA	5HT	5HIAA
6 Days						
Diet I	272	245	77	69	611	76
Diet II	205*	182	68	75	425*	51***
Diet III	275	179	155	74	363**	35***
Diet IV	277	241	145	100	565	73
sem (6 d.f.)	26	27	29	82	38	3
14 Days						
Diet I	214	175	111	119	511*	80
Diet II	141	154	156	59*	285	45
Diet III	248	201	135	116	396	42*
Diet IV	276	227	117	81	483	81
sem (6 d.f.)	22	44	61	17	45	11
21 Days						
Diet I	254	217	213	87	551	112
Diet II	197	205	114	66	467	87
Diet III	272	229	116	74	524	83
Diet IV	245	211	279	97	561	203
sem (6 d.f.)	19	22	54	16	46	38

Values significantly different from control values *P<0.05, **P<0.01, ***P<0.001

I Control diet
II Control+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg)
III Control+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg) +phenylalanine (12g/kg)
IV Control+leucine (60g/kg)+isoleucine (34.7g/kg)+valine (40g/kg) +phenylalanine (12g/kg)+tryptophan (8g/kg)

phenylalanine, was significantly greater than in chicks receiving diets incorporating the BCAA mixture alone P<0.05) or in combination with phenylalanine (P<0.01). Similarly, brain concentrations of 5HIAA were significantly higher in birds consuming either the control diet or that to which all three additions had been made than in those fed the other two diets (P<0.001). In addition, chicks fed the diet incorporating the BCAA mixture and additional phenylalanine had significantly lower brain concentrations of 5HIAA than those fed the diet supplemented with only the BCAA (P<0.01).

In chicks which had been consuming the experimental diets for fourteen days, brain concentrations of NE were significantly higher in those receiving both the BCAA mixture and additional phenylalanine in comparison with those fed the diet containing the BCAA mixture alone (P<0.05). No significant differences were observable in the concentrations of DA and DOPAC in the brains of birds fed the different diets, while those consuming the diet incorporating the BCAA mixture alone showed HVA concentrations which were below those of chicks fed the control diet (P<0.05).

Chicks fed either the unsupplemented diet or that into which tryptophan, phenylalanine and the BCAA mixture had all been incorporated showed a higher brain concentration of 5HT than those receiving the diet containing the BCAA mixture alone (P<0.05), and a higher concentration of 5HIAA than those consuming the diet incorporating both the BCAA mixture and phenylalanine (P<0.05).

After twenty-one days of feeding the experimental diets, chicks which had received the diet supplemented only with the BCAA mixture had significantly lower brain concentrations of NE than those fed that diet into which both the BCAA mixture and additional

phenylalanine had been incorporated ($P<0.05$). No significant difference in the brain concentrations of any other measured neurotransmitter or metabolite in birds fed the various diets was observed.

ii. Effects of age and length of feeding period

The brain concentration of NE in chicks fed the control diet for fourteen days was significantly less than that of those which had received the same diet for only six days ($P<0.01$) or for the whole of the feeding period ($P<0.05$). Similarly, birds consuming the diet incorporating the BCAA mixture alone had a greater brain concentration of NE after six days of feeding than after fourteen ($P<0.05$). The HVA concentration in the brains of chicks receiving the diet containing both the BCAA mixture and a supplement of phenylalanine alone was greater after fourteen days of the experimental period than after only six ($P<0.01$). At the end of the period of feeding, the brain concentration of 5HIAA in chicks fed the diet incorporating only the BCAA mixture was greater than that of birds which had consumed the same diet for only fourteen days ($P<0.05$). Chicks which had received the diet containing both the BCAA mixture and a supplement of phenylalanine alone also had a greater brain concentration of 5HIAA after twenty-one days of feeding than after fourteen ($P<0.05$). The brain concentrations of both HVA ($P<0.01$) and 5HIAA ($P<0.05$) were significantly higher in chicks which had consumed the diet containing the BCAA mixture alone for the entire experimental period than in those fed that diet for only six days.

iii. Neurotransmitter concentrations and food intake

Chick brain concentrations of NE, DA and 5HT were not at any time correlated with the total quantity of food consumed. After fourteen days of feeding the diets, the brain NE concentration was positively correlated with the amount of protein ingested ($r=0.589$,

P<0.05), as was the brain concentration of 5HT at the end of the experimental period (r=0.515, P<0.05). At no other times were the chick brain concentrations of NE, DA or 5HT correlated with the quantity of either protein or AME(N) consumed. The concentration of NE in the chick brain did however correlate positively with the amount of tyrosine+phenylalanine ingested after six (r=0.637, P<0.01), fourteen (r=0.769, P<0.001) and twenty-one days (r=0.558, P<0.05) of feeding. On the sixth (r=0.736, P<0.01) and fourteenth (r=0.594, P<0.05) days of the experimental period, the chick brain concentration of 5HT was positively correlated with the ratio of the dietary content of tryptophan to the combined concentrations of leucine+isoleucine+valine+tyrosine+phenylalanine in the diet. No other correlations were observed between brain neurotransmitter concentrations and the intake of their precursors alone or relative to that of their LNAA competitors for brain uptake.

3.13 Experiment 12. The effect of additional tryptophan on chicks fed high concentrations of dietary phenylalanine

a. Dietary nitrogen and AME(N) content

As shown in Table 31, the nitrogen contents of the diets presented during this investigation did not differ greatly, that of the control diet perhaps being slightly larger than that of the others.

Few significant differences in the AME(N) content of the experimental diets were observable. That of the diet supplemented with phenylalanine at 20g/kg and tryptophan at 4g/kg was significantly higher than that of the control diet or that to which phenylalanine alone at 20g/kg had been added (P<0.05). The diet incorporating a sole phenylalanine supplement of 40g/kg had an AME(N) content greater than that of the control diet (P<0.01), that supplemented with tryptophan alone (P<0.05), or that supplemented only with phenylalanine at 20g/kg

Table 31. Determined nitrogen and AME(N) contents of diets fed during investigation of the effect of additional tryptophan on a dietary excess of phenylalanine

	DIET FED							
	I	II	III	IV	V	VI	VII	VIII
NITROGEN CONTENT (g/kg dry matter)	34.91	34.11	34.11	34.06	33.83	34.15	3.62	34.40
AME(N) (MJ/kg dry matter)	14.80	14.93	14.74	15.22	15.39*	5.62**	15.12	15.25

sem (21 d.f.)=0.19

Values significantly different from those of control diet *P<0.05, **P<0.01, ***P<0.001

I Control diet having low content of tryptophan
II Control+tryptophan (1g/kg)
III Control+phenylalanine (20g/kg)
IV Control+phenylalanine (20g/kg)+tryptophan (2g/kg)
V Control+phenylalanine (20g/kg)+tryptophan (4g/kg)
VI Control+phenylalanine (40g/kg)
VII Control+phenylalanine (40g/kg)+tryptophan (2g/kg)
VIII Control+phenylalanine (40g/kg)+tryptophan (4g/kg)

(P<0.01).

b. Growth and food intake (Figs. 35. and 36.)

Chicks receiving the diet supplemented only with tryptophan at 1g/kg constantly had a much greater weight gain and food intake than those fed the tryptophan-deficient control diet (P<0.001). However after only two days of feeding, birds consuming the diet incorporating phenylalanine alone at 20g/kg showed very significantly lower levels of weight gain and food intake than those fed either control diet (P<0.001). The depression in food intake was maintained at this very high level of significance throughout the experimental period, while the reduction in weight gain attained significance only at the level of P<0.01 on the twelfth and thirteenth days of feeding. Both the weight gain and food intake of chicks fed the diet supplemented only with phenylalanine at 20g/kg were constantly very significantly depressed in comparison with birds fed that supplemented with tryptophan alone (P<0.001).

The concomitant addition of phenylalanine at 20g/kg and tryptophan at 2g/kg to the diet resulted in the weight gain of chicks fed this diet being raised above that of those fed the control diet (P<0.001). On days two and four (P<0.01) and thereafter (P<0.001), the food intake of birds receiving the former diet was also significantly greater than that of those fed the control diet. Both the weight gain and food intake of chicks fed the diet incorporating phenylalanine at 20g/kg and tryptophan at 2g/kg were constantly significantly less than those of birds receiving the diet supplemented only with tryptophan and greater than those of chicks fed the diet supplemented with phenylalanine alone at 20g/kg (P<0.001).

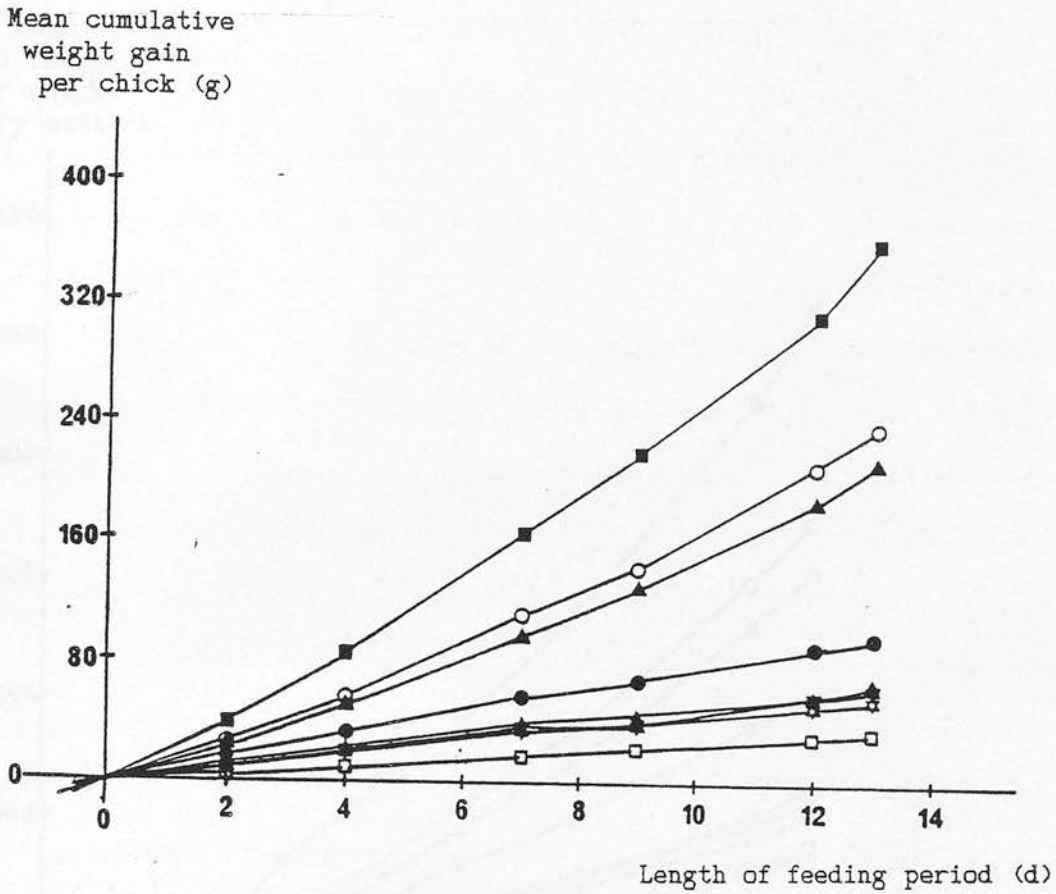


Fig. 35. Weight gain of chicks fed excess phenylalanine alone or with additional tryptophan

●	I	Control diet having low content of tryptophan
■	II	Control+tryptophan (1g/kg)
✱	III	Control+phenylalanine (20g/kg)
▲	IV	Control+phenylalanine (20g/kg)+tryptophan (2g/kg)
○	V	Control+phenylalanine (20g/kg)+tryptophan (4g/kg)
□	VI	Control+phenylalanine (40g/kg)
✡	VII	Control+phenylalanine (40g/kg)+tryptophan (2g/kg)
△	VIII	Control+phenylalanine (40g/kg)+tryptophan (4g/kg)

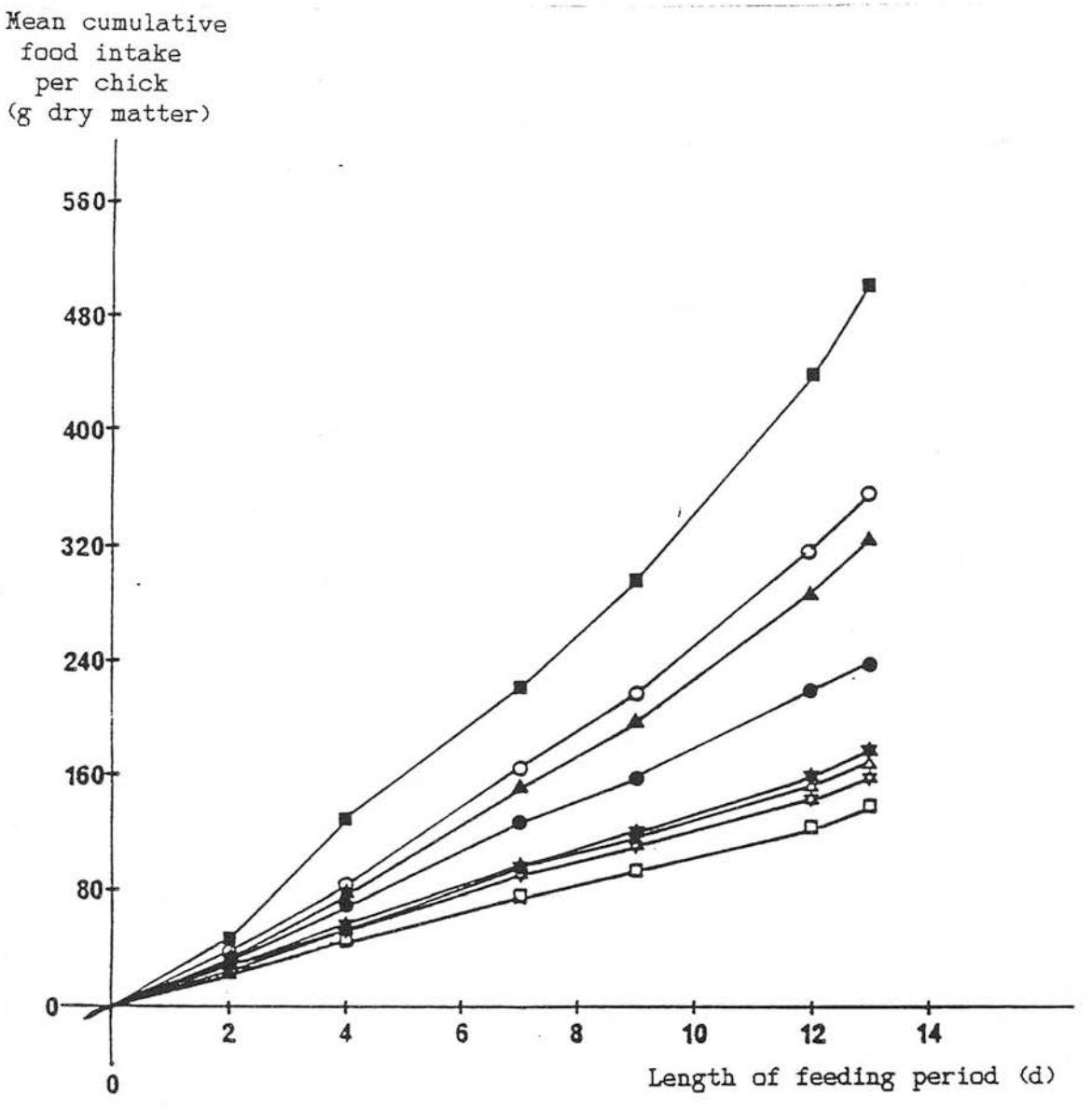


Fig. 36. Food intake of chicks fed excess phenylalanine alone or with additional tryptophan

●	I	Control diet having low content of tryptophan
■	II	Control+tryptophan (1g/kg)
✱	III	Control+phenylalanine (20g/kg)
▲	IV	Control+phenylalanine (20g/kg)+tryptophan (2g/kg)
○	V	Control+phenylalanine (20g/kg)+tryptophan (4g/kg)
□	VI	Control+phenylalanine (40g/kg)
✡	VII	Control+phenylalanine (40g/kg)+tryptophan (2g/kg)
△	VIII	Control+phenylalanine (40g/kg)+tryptophan (4g/kg)

Birds consuming the diet incorporating phenylalanine at 20g/kg and tryptophan at 4g/kg had a level of weight gain and food intake which were very much greater than those of birds fed the control diet or that incorporating only phenylalanine at 20g/kg, but less than those of birds receiving the diet supplemented with tryptophan alone ($P<0.001$). From the seventh day of the feeding period onwards, the weight gain and food intake of chicks fed the diet incorporating phenylalanine at 20g/kg and tryptophan at 4g/kg were significantly greater than those of birds consuming the diet containing the same amount of phenylalanine and half the quantity of tryptophan ($P<0.05$).

Chicks fed the diet incorporating phenylalanine alone at a concentration of 40g/kg, showed a very highly significant depression in growth and food intake compared with those receiving the control diet, that supplemented only with tryptophan, or diets incorporating phenylalanine at 20g/kg and tryptophan at either 2g/kg or 4g/kg ($P<0.001$). On the ninth day of the feeding period ($P<0.05$) and at all other times of measurement ($P<0.01$), the weight gain of birds fed the diet containing this maximum amount of phenylalanine alone was significantly less than that of those receiving the diet incorporating half the amount of this amino acid alone. The food intake of chicks receiving the former diet was also below that of those fed the latter on days two ($P<0.001$), four ($P<0.01$), seven and nine ($P<0.001$), twelve and thirteen ($P<0.01$) of the experiment.

Both the weight gain and food intake of chicks fed the diet containing additional phenylalanine at 40g/kg and tryptophan at 2g/kg remained significantly below those of birds fed the control diet, that supplemented only with tryptophan, or those diets incorporating phenylalanine at 20g/kg with tryptophan at 2g/kg or 4g/kg ($P<0.001$).

However, no significant difference in weight gain or food intake was observable between chicks fed the diet containing phenylalanine alone at 20g/kg and those receiving the diet incorporating twice as much phenylalanine with additional tryptophan at 2g/kg. Birds consuming the latter diet showed a greater weight gain than those fed the diet incorporating the same amount of phenylalanine without supplemental tryptophan, the difference being significant on days four and seven of the experimental period ($P<0.01$) and at all other times of measurement ($P<0.05$). The food intake of chicks fed the diet incorporating both phenylalanine at 40g/kg and tryptophan at 2g/kg was initially significantly above that of those fed the diet containing the same amount of phenylalanine alone ($P<0.05$), but there was no significant difference in the food intake of birds fed these two diets on the twelfth and thirteenth days of feeding.

Chicks receiving the diet incorporating phenylalanine at 40g/kg and tryptophan at 4g/kg had significantly lower levels of weight gain ($P<0.01$) and food intake ($P<0.001$) than those fed the control diet. Both the weight gain and food intake of chicks fed the former diet were also very significantly less than those of birds consuming the diet supplemented only with tryptophan or diets incorporating phenylalanine at 20g/kg and tryptophan at 2g/kg or 4g/kg ($P<0.001$). However, neither the weight gain nor food intake of birds fed the diet incorporating phenylalanine at 40g/kg and tryptophan at 4g/kg were significantly different from those of chicks fed diets incorporating phenylalanine alone at 20g/kg or both phenylalanine at 40g/kg and tryptophan at 2g/kg. In comparison with chicks receiving the diet incorporating phenylalanine alone at 40g/kg, the weight gain of those fed the diet which was simultaneously supplemented with tryptophan at 4g/kg was significantly

increased on the second day of feeding ($P<0.001$) and thereafter ($P<0.01$). Birds fed the latter diet also had a greater food intake than those receiving the former, this being apparent on the second ($P<0.01$), and seventh ($P<0.01$) days of feeding and at all other times of measurement ($P<0.05$).

c. Neurotransmitter concentrations

i. Dietary effects

Determinations of E and HVA in the brains of chicks fed the experimental diets were not made owing to the response ratios measured differing from those of injected standards. As indicated in Table 32, no significant difference in brain concentrations of NE or DA was apparent between the groups of chicks consuming the various diets. The concentration of DOPAC in chicks fed the diet having the maximum incorporated concentrations of both phenylalanine and tryptophan was however significantly greater than in those receiving the control diet, that supplemented only with tryptophan, or that containing phenylalanine at 20g/kg and tryptophan at 4g/kg ($P<0.001$). Birds consuming diets to which phenylalanine had been added at a concentration of 20g/kg either alone or with a tryptophan supplement of 2g/kg, also had a brain concentration of DOPAC which was below that of those receiving the maximum incorporated concentrations of each of these amino acids ($P<0.01$). Again, the brain DOPAC concentration of chicks fed additional phenylalanine at a dietary concentration of 40g/kg was significantly less than that of those fed the latter diet ($P<0.05$). Birds receiving the diet supplemented only with tryptophan had a significantly lower brain DOPAC concentration than that of chicks fed additional phenylalanine at 20g/kg ($P<0.05$) or 40g/kg ($P<0.01$) in the presence or absence of

Table 32. Concentrations of neurotransmitters and metabolites in the brains of chicks fed excess phenylalanine alone or with additional tryptophan

Diet	Concentration of compound (ng/g fresh wt. brain tissue)				
	NE	DA	DOPAC	5HT	5HIAA
I	139	207	69	288	35
II	135	160	40	469*	80***
III	126	220	78	261	34
IV	142	179	82	402	53*
V	186	228	67	527*	69***
VI	190	240	101	219	19*
VII	147	240	97	375	40
VIII	127	225	141***	350	45
sem (21 d.f.)	27	40	12	61	6

Values significantly different from those of birds fed the control diet
*P<0.05, **P<0.01, ***P<0.001

I	Control diet having low content of tryptophan
II	Control+tryptophan (1g/kg)
III	Control+phenylalanine (20g/kg)
IV	Control+phenylalanine (20g/kg)+tryptophan (2g/kg)
V	Control+phenylalanine (20g/kg)+tryptophan (4g/kg)
VI	Control+phenylalanine (40g/kg)
VII	Control+phenylalanine (40g/kg)+tryptophan (2g/kg)
VIII	Control+phenylalanine (40g/kg)+tryptophan (4g/kg)

tryptophan at 2g/kg. No significant difference in the brain concentrations of DA in chicks fed the various diets was apparent.

The concentration of 5HT in the brains of chicks fed the control diet was significantly less than that of those receiving additional tryptophan alone or both phenylalanine at 20g/kg and tryptophan at 4g/kg ($P<0.05$). Those fed the diet supplemented only with tryptophan also had a greater concentration of 5HT than chicks consuming diets incorporating phenylalanine alone at a concentration of 20g/kg ($P<0.05$) or 40g/kg ($P<0.01$). An increased brain concentration of 5HT was apparent in chicks receiving the diet containing additional phenylalanine at 20g/kg with tryptophan at 4g/kg, in comparison with those fed either diet containing excess phenylalanine alone($P<0.01$). Birds consuming the diet incorporating phenylalanine alone at a concentration of 40g/kg also had significantly lower brain concentrations of 5HT than those fed additional phenylalanine at 20g/kg supplemented with tryptophan at 2g/kg ($P<0.05$).

Chicks receiving the diet supplemented only with tryptophan showed a higher brain concentration of 5HIAA than those fed the diet incorporating phenylalanine at 20g/kg and tryptophan at 2g/kg ($P<0.01$), or any of the other diets but that concomitantly supplemented with phenylalanine at 20g/kg and tryptophan at 4g/kg ($P<0.001$). Birds fed the latter diet also had greater brain 5HIAA concentrations than those consuming that incorporating the same amount of phenylalanine but half the quantity of tryptophan ($P<0.05$), either of the tryptophan-supplemented diets containing phenylalanine at 40g/kg ($P<0.01$), or any of the other diets except that supplemented only with tryptophan ($P<0.001$). A significantly higher brain concentration of 5HIAA was apparent in chicks fed the diet to which phenylalanine at a

concentration of 20g/kg and tryptophan at 2g/kg had been added, in comparison with those fed the control diet or that incorporating phenylalanine alone at 20g/kg ($P<0.05$), or phenylalanine alone at 40g/kg ($P<0.001$). Chicks fed this latter diet showed concentrations of 5HIAA in the brain which were also below those measured in birds receiving the control diet ($P<0.05$) or diets having the maximum incorporated concentration of phenylalanine with supplementary tryptophan at either 2g/kg ($P<0.05$) or 4g/kg ($P<0.01$).

ii. Neurotransmitter concentrations and food intake

Chick brain concentrations of NE and DA were not correlated with the total amount of food consumed or the quantities of protein, AME(N) or tyrosine+phenylalanine ingested. The concentration of 5HT in the chick brain was positively correlated with the amount of food ingested ($r=0.514$, $P<0.01$) and the quantity of protein ($r=0.508$, $P<0.01$) or AME(N) ($r=0.523$, $P<0.01$) consumed. It was also significantly positively correlated with the quantity of tryptophan ingested ($r=0.615$, $P<0.001$).

3.14 Experiment 13. The effect of additional phenylalanine on chicks fed high concentrations of dietary tryptophan

a. Dietary nitrogen and AME(N) content

Some variation in nitrogen content between the experimental diets presented was observed (Table 33), this tending to increase with the phenylalanine content of the diet except at a level of phenylalanine supplementation of 20g/kg when a slight decrease was apparent. Addition of tryptophan at a concentration of 30g/kg appeared to slightly reduce dietary nitrogen content.

The AME(N) of the diet containing additional phenylalanine alone at a concentration of 8g/kg was significantly higher than that of

Table 33. Nitrogen and AME(N) content of diets formulated during investigation of the effect of additional phenylalanine on a dietary excess of tryptophan.

	DIET FED					
	I	II	III	IV	V	VI
NITROGEN CONTENT (g/kg dry matter)	27.28	28.84	26.74	27.15	27.78	25.87
AME(N) (MJ/kg dry matter)	14.54	15.05*	14.68	14.68	14.58	15.38**

sem (15 d.f.)=0.15

Values significantly different from that of control diet *P<0.05, **P<0.01

I	Control diet formulated to have a low content of phenylalanine
II	Control+phenylalanine (8g/kg)
III	Control+tryptophan (30g/kg)
IV	Control+tryptophan (30g/kg)+phenylalanine (8g/kg)
V	Control+tryptophan (30g/kg)+phenylalanine (12g/kg)
VI	Control+tryptophan (30g/kg)+phenylalanine (20g/kg)

either the control diet or that supplemented with tryptophan at 30g/kg and phenylalanine at 12g/kg ($P<0.05$) (Table 33). The diet incorporating both excess tryptophan and the highest concentration of supplemental phenylalanine had a greater AME(N) than the control diet ($P<0.001$) or any of the other diets containing excess tryptophan ($P<0.01$).

b. Growth and food intake (Figs. 37. and 38.)

Chicks receiving the diet incorporating phenylalanine at 8g/kg had a significantly greater weight gain and food intake than those fed the control diet, this being apparent after only two days of feeding ($P<0.001$). The weight gain of chicks fed the diet incorporating excess tryptophan was severely reduced below that of birds receiving the control diet, the difference in weight gain being very highly significant for most of the feeding period ($P<0.001$) and attaining significance at the level of $P<0.01$ at the end of the experiment. The fall in food intake which accompanied this reduction in weight gain attained significance at a very high level ($P<0.001$) initially and at lower levels on the eighth ($P<0.01$) and twelfth ($P<0.05$) days of feeding. Both the weight gain and food intake of birds receiving the diet incorporating excess tryptophan alone were very much less than those of chicks fed the diet supplemented with phenylalanine alone ($P<0.001$).

In comparison with birds consuming the control diet, the weight gain of chicks fed the diet containing both excess tryptophan and supplemental phenylalanine at 8g/kg was significantly reduced on day two of the feeding period ($P<0.001$) and thereafter ($P<0.01$). The food intake of birds fed the latter diet was also below that of those receiving the former on days two and four ($P<0.001$), six and eight ($P<0.01$) and twelve ($P<0.05$) of feeding. Again, chicks fed the diet incorporating excess tryptophan together with phenylalanine at 8g/kg had

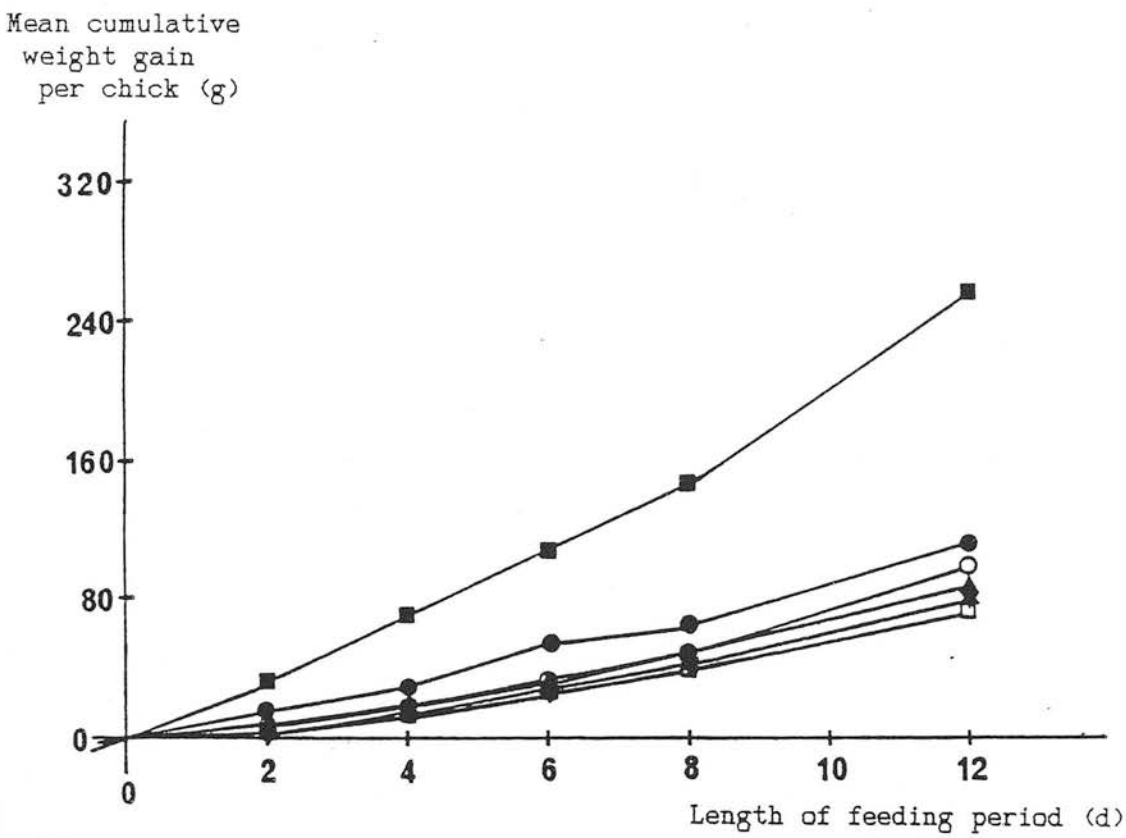


Fig. 37. Weight gain of chicks fed excess tryptophan alone and with additional phenylalanine

●	I	Control diet formulated to have a low content of phenylalanine
■	II	Control+phenylalanine (8g/kg)
★	III	Control+tryptophan (30g/kg)
▲	IV	Control+tryptophan (30g/kg)+phenylalanine (8g/kg)
○	V	Control+tryptophan (30g/kg)+phenylalanine (12g/kg)
□	VI	Control+tryptophan (30g/kg)+phenylalanine (20g/kg)

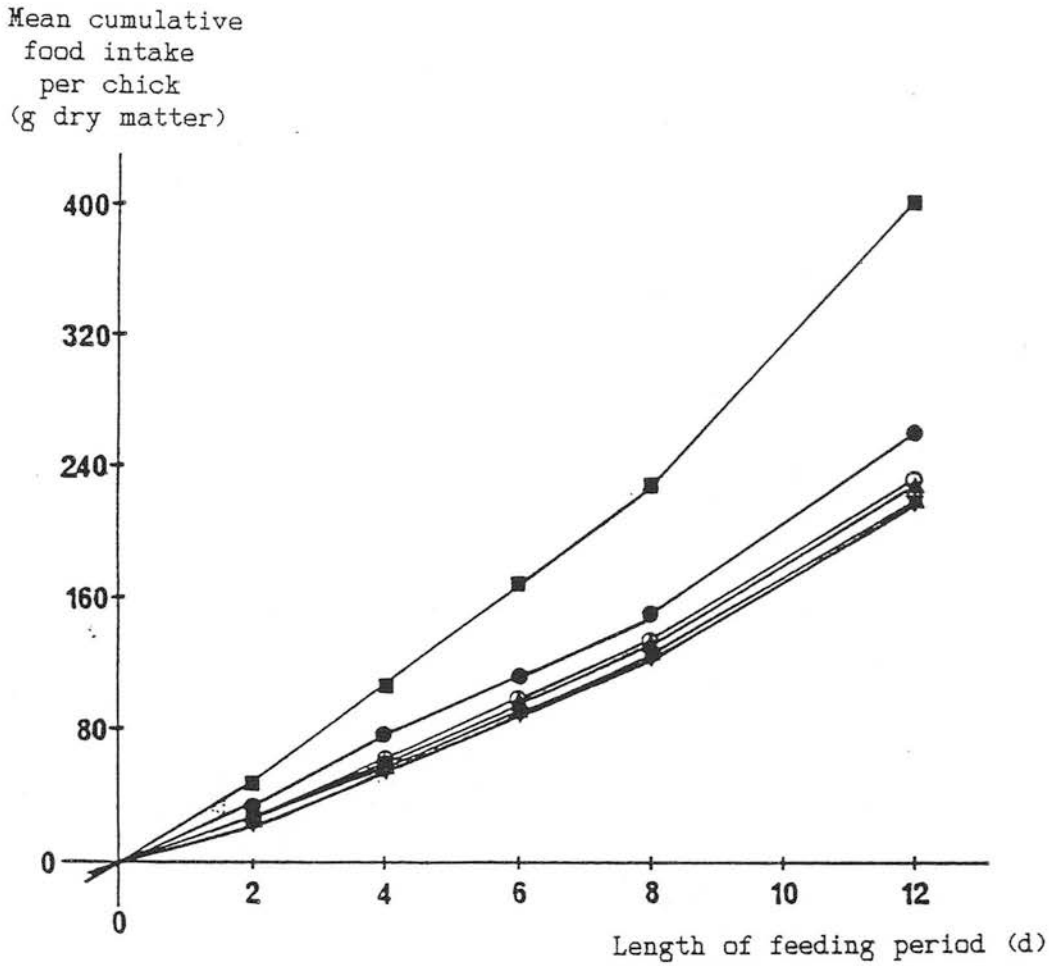


Fig. 38. Food intake of chicks fed excess tryptophan alone and with additional phenylalanine

●	I	Control diet formulated to have a low content of phenylalanine
■	II	Control+phenylalanine (8g/kg)
✱	III	Control+tryptophan (30g/kg)
▲	IV	Control+tryptophan (30g/kg)+phenylalanine (8g/kg)
○	V	Control+tryptophan (30g/kg)+phenylalanine (12g/kg)
□	VI	Control+tryptophan (30g/kg)+phenylalanine (20g/kg)

very much lower levels of weight gain and food intake than those receiving the diet containing the same quantity of phenylalanine in the absence of excess tryptophan ($P<0.001$). Neither the weight gain nor the food intake of chicks fed the diet containing both excess tryptophan and additional phenylalanine at 8g/kg was significantly different from that of birds receiving the diet containing excess tryptophan alone, although there was a tendency for those fed additional phenylalanine to have a somewhat improved level of growth.

On the second day of the experimental period, the weight gain of chicks fed the diet incorporating both excess tryptophan and supplemental phenylalanine at 12g/kg was significantly below that of birds receiving the control diet ($P<0.001$). The level of significance of this depression in growth subsequently fell to $P<0.01$, and on the final day of feeding there was no significant difference in weight gain between chicks fed these two diets. The food intake of birds receiving the control diet was greater than that of those fed the diet containing excess tryptophan and phenylalanine at 12g/kg on day two of feeding ($P<0.01$), but this difference had fallen in significance by day eight ($P<0.05$) and was not significant on the final day of the experiment. In comparison with birds fed the diet supplemented only with phenylalanine, the weight gain and food intake of those receiving the diet incorporating excess tryptophan together with phenylalanine at 12g/kg were each continually reduced ($P<0.001$). No significant difference was apparent between either the weight gain or the food intake of birds fed the diets containing excess tryptophan alone or with phenylalanine at 8g/kg, and that of those receiving the same amount of tryptophan with phenylalanine at 12g/kg, although those fed the greater amount of phenylalanine tended to have the greater growth.

Birds receiving the diet incorporating both excess tryptophan and additional phenylalanine at 20g/kg had a significantly lower weight gain than those fed either the control diet or that supplemented with phenylalanine alone ($P<0.001$). The food intake of chicks fed the diet containing excess tryptophan and the greatest amount of phenylalanine was also below that of those consuming the control diet from the second day of the experiment onwards ($P<0.001$), the difference attaining lower levels of significance on the eighth ($P<0.01$) and twelfth ($P<0.05$) days of feeding. In comparison with birds fed the diet supplemented only with phenylalanine, the food intake of birds receiving that containing the both excess tryptophan and the largest supplement of phenylalanine was always very significantly reduced ($P<0.001$).

There was no significant difference apparent between the food intake of birds fed the diet containing both excess tryptophan and phenylalanine at 20g/kg, and that of those fed diets containing the same amount of tryptophan alone or with smaller phenylalanine supplements. Chicks receiving diets containing excess tryptophan alone or with phenylalanine at 8g/kg at no time had a significantly different weight gain than those fed the diet incorporating both excess tryptophan and the largest phenylalanine supplement, although birds fed the greatest amount of phenylalanine tended to have a lower weight gain than those fed the other two diets. On the final day of the feeding period, the weight gain of birds fed the diet containing both excess tryptophan and additional phenylalanine at 20g/kg was significantly less than that of those receiving the diet incorporating the same quantity of tryptophan but a phenylalanine supplement of only 12g/kg ($P<0.01$).

c. Neurotransmitter concentrations

i. Dietary effects

Measurement of E, DOPAC and HVA concentrations in the brains of chicks fed the experimental diets was prone to error due to contaminant interference and these values have not therefore been reported. As shown in Table 34, the brain NE concentration of birds consuming the diet containing excess tryptophan with a supplement of phenylalanine at a concentration of 8g/kg was significantly higher than that of those fed the control diet ($P<0.01$). Chicks receiving the control diet showed a lower brain concentration of DA than those fed the diets containing excess tryptophan alone ($P<0.05$) or with additional phenylalanine at any of the supplied concentrations ($P<0.01$). Similarly, the concentration of DA in the brains of birds consuming the diet supplemented only with phenylalanine was significantly below that of those fed diets incorporating excess tryptophan alone ($P<0.05$) or with additional phenylalanine at concentrations of 8g/kg, ($P<0.05$), 12g/kg ($P<0.05$) or 20g/kg ($P<0.01$).

The brain concentrations of 5HT in chicks receiving excess tryptophan in the absence of additional phenylalanine or with phenylalanine supplements of 8g/kg or 20g/kg, were significantly higher than those of birds fed either the control diet or that supplemented only with phenylalanine ($P<0.05$). In the brains of chicks consuming the diet incorporating excess tryptophan alone, the concentration of 5HIAA was greater than that measured for those fed any of the diets other than that containing the same amount of tryptophan and supplemented with phenylalanine at a concentration of 8g/kg ($P<0.01$). In a similar manner, chicks fed the latter diet also showed a significantly greater brain concentration of 5HIAA than those receiving the control diet or

Table 34. Concentrations of neurotransmitters and metabolites in the brains of chicks fed excess tryptophan alone and with additional phenylalanine

Diet	Concentration of compound (ng/g fresh wt. brain tissue) NE	DA	5HT	5HIAA
Diet I	226	248	772	105
Diet II	292	268	756	99
Diet III	300	396*	1328*	323**
Diet IV	347**	425**	1401*	331**
Diet V	284	426**	1233	88
Diet VI	283	456**	1405*	136
sem (15 d.f.)	29	39	160	41

Values significantly different from those of chicks fed control diet
*P<0.05, **P<0.01, ***P<0.001

I	Control diet formulated to have a low content of phenylalanine
II	Control+phenylalanine (8g/kg)
III	Control+tryptophan (30g/kg)
IV	Control+tryptophan (30g/kg)+phenylalanine (8g/kg)
V	Control+tryptophan (30g/kg)+phenylalanine (12g/kg)
VI	Control+tryptophan (30g/kg)+phenylalanine (20g/kg)

that supplemented with phenylalanine alone ($P<0.01$), or those incorporating excess tryptophan with phenylalanine supplements of 12g/kg ($P<0.001$) or 20g/kg ($P<0.01$)

ii. Neurotransmitter concentrations and food intake

The brain concentration of NE in chicks fed the experimental diets was not correlated with the quantity of food consumed or the amounts of protein, AME(N) or tyrosine+phenylalanine ingested. Negative correlations were apparent between the brain concentration of DA and the amounts of food as a whole ($r=0.576$, $P<0.01$) or protein ($r=0.573$, $P<0.01$) or AME(N) ($r=0.551$, $P<0.01$) consumed by the bird. The chick brain concentration of 5HT was correlated with food intake ($r=0.533$, $P<0.05$), protein intake ($r=0.536$, $P<0.01$) and AME(N) intake ($r=0.514$, $P<0.05$). It was also significantly positively correlated with the amount of tryptophan ingested ($r=0.662$, $P<0.001$).

CHAPTER FOUR

DISCUSSION

4.1 Determination of neurotransmitter concentrations by HPLC

The choice of an appropriate HPLC method for the separation of NE, E, DA and 5HT and a selection of their metabolites required the study and modification of several reported procedures. However, the time required for this was offset by the consequent ability to quantitatively determine several of the components of an extract of the chick brain in a relatively short time. The advent of the use of HPLC in neurotransmitter analysis is quite recent and the earliest reports of such methods were in the determination of the concentrations of such compounds and metabolites in the plasma, urine, or rat brain (Davis *et al.*, 1979; Hjemdahl *et al.*, 1979; Krstulovic and Powell, 1979; Mefford and Barchas, 1980). The employment of such procedures in the study of the effects of the diet on brain neurotransmitter concentrations in the rat does not as yet appear to be common, but has been reported (Peters and Harper, 1981, 1985). No work appears to be currently available which has used HPLC to determine the brain concentrations of NE, E, DA, 5HT or any of the metabolites of these neurotransmitters in the chick. The effects of variations in the composition of the diet on the concentrations of such neurotransmitter compounds in the chick brain do not appear to have been studied by any method. As the biochemical basis of reported amino acid interactions, and the mechanism by which distortions in the dietary amino acid pattern are recognised by the central nervous system remain obscure, the availability of HPLC techniques and their ability to quantitate very small amounts of many substances should provide a large amount of information regarding the response of the chick to its nutritional environment.

4.2 Experiment 1. Feeding of the starter diet

The mean brain concentrations of 5HT of 731ng/g and 769ng/g fresh brain tissue measured for birds receiving the starter diet for the periods indicated (Table 13), compare reasonably well with the concentrations of the order of 1μg/g reported by Brodie and Bogdanski (1964) and 860-880 ng/g by Pscheidt and Tamimie (1966) for broiler chicks fed a maize-soyabean based diet. However, reported chick brain concentrations of NE of 600ng/g (Brodie and Bogdanski, 1964), over 800ng/g (Pscheidt and Tamimie, 1966) and approximately 1.4μg/g (Callingham and Sharman, 1970) are rather higher than the concentrations of about 260ng/g determined here for birds of similar ages. It is possible that these differences are due to the differences in amino acid content of the diets fed. This cannot however be confirmed because of a lack of data regarding the precise content of amino acids or ingredients in the diets employed. Alternatively, it is also possible that continued selection of broiler chickens has resulted in the strain employed in recent experiments having lower brain concentrations of NE. The existence of strain differences was indeed reported by Pscheidt and Tamimie (1966), chicks of a non-broiler strain having brain NE concentrations of less than 600ng/g fresh brain tissue.

The very few significant differences observed in the brain concentrations of the neurotransmitters and metabolites measured in chicks killed at the different times, indicated that there was little effect of diurnal variations over the period during which all chicks of subsequent experiments were likely to be killed. Only at the fourteenth day of feeding was a depression in the brain concentration of 5HT seen in the early afternoon, possibly due to some degree of diurnal variation. A fall in rat brain 5HT and 5HIAA concentrations at night has been

reported by Fernstrom *et al.* (1985), there being a tendency for these compounds to decrease gradually in concentration during the day. However, no report of a fall in, and subsequent restoration of, the brain concentration of 5HT during the day has been reported. The difference in effects could perhaps be attributed to the great difference in the length of the dark period, this being of only two hours duration in the current study, and twelve hours during the investigations of Fernstrom *et al.* (1985). It is unfortunate that due to unforseeable circumstances measurements could not be made at three time periods at day twenty-one, since comparison of three concentrations of 5HT in particular would have been of interest. It does nevetheless appear that the concentration of 5HT in the chick brain may not be constant over the time interval during which heads might be collected. As decapitation of all the chicks of a large experiment required approximately one hour, the process in further experiments was carried out as quickly as possible and replicate by replicate, in order that introduction of error due to variation in chick brain neurotransmitter or metabolite concentrations with time might be minimised.

It is apparent that there is initially an increase in the chick brain concentrations of NE, DA and 5HT with age. Birds fed the diet for the entire experimental period did not however have significantly greater brain concentrations of these compounds than those which had received the starter diet for seven days less. This suggests either that there is not a continual increase in the concentrations of these compounds with age and the length of the feeding period, or that the interval between these two times of sampling is insufficient to show such an effect. The reason for the transient increase in brain concentrations of DOPAC after feeding the starter diet for fourteen days

beyond the usual seven-day period is unclear, this effect being lost by the end of the experiment.

4.3 Experiment 2. The lysine-arginine antagonism

a. Dietary nitrogen content

As the diet having the greatest nitrogen content (Table 14) supported the least chick growth and food intake, it is unlikely that observed differences in growth and food intake of chicks fed the experimental diets were due to the small differences measured in the dietary nitrogen content.

b. Growth and food intake (Figs. 14 and 15)

Chicks fed the experimental diets exhibited the classical changes in growth and food intake caused by the phenomenon of lysine-arginine antagonism, consumption of excess dietary lysine causing a depression in growth and food intake which was greatly alleviated by additional arginine. Such effects are comparable to those reported by many groups of workers (Boorman and Fisher, 1965; D'Mello and Lewis, 1970a; also see Chapter 1). The accompanying effects on brain concentrations of NE, DA, 5HT and their metabolites do not however appear to have been studied previously.

c. Neurotransmitter concentrations (Table 15)

i. Effects of diet and food intake

It appears that the addition of excess lysine alone to the diet tends within the first four days of feeding to reduce the brain concentrations of NE, DA and 5HIAA in the chick, and that these reductions are prevented by the concomitant addition of arginine although differences in the concentrations of these compounds are only significant when comparing the effects of these two diets. The existence of an effect of arginine on 5HT metabolism is supported by the greater

brain concentration of 5HT in chicks fed the diet supplemented only with arginine than in those receiving excess lysine alone for eight days or the control diet for the full experimental period. Such changes in the brain concentrations of 5HT and 5HIAA are consistent with supplemental arginine causing an increase in the synthesis of 5HT which may then be followed by a rise in the breakdown of this neurotransmitter and an increased brain concentration of 5HIAA. The effect of excess lysine would appear to be a reduction in 5HT synthesis, its degradation being decreased in order to maintain its brain concentration.

The increased brain concentration of HVA in birds fed the diet containing excess lysine alone, together with the fall in brain DA concentrations, would indicate a lysine-induced increase in the breakdown of this neurotransmitter. While the brain HVA concentration is increased further in chicks simultaneously fed an arginine supplement, the accompanying restoration of DA concentrations is consistent with a rise in both the synthesis and the degradation of DA.

Neither lysine nor arginine is transported into the brain by the system which takes up the LNAA precursors of NE, DA and 5HT (Olendorf and Szabo, 1976). The observed effects of these amino acids on brain neurotransmitter concentrations cannot therefore be attributed directly to changes in precursor availability due to altered competition for brain uptake. It is perhaps possible that at such excessive dietary concentrations, lysine could begin to compete to a significant extent with the LNAA transport system and hence reduce the brain uptake of tryptophan, tyrosine and phenylalanine. A mechanism by which arginine might alleviate such effects is however unclear. Possible inhibitory effects of lysine on neurotransmitter synthesis within the brain cannot be ruled out. As lysine and arginine compete with each other for their

uptake into the brain, the alleviation of lysine-caused changes in neurotransmitter metabolism by the addition of arginine could then be explained. However, if excessive lysine was able to exert such effects within the brain, more extensive differences in the brain neurotransmitter concentrations of chicks fed the different diets might have been expected.

The positive correlation of the chick brain concentrations of 5HT after eight days of feeding, with the level of food and hence also tryptophan intake, provides some indication that the synthesis of 5HT may be influenced by precursor availability. The correlation is however weak, and is not shown at other sampling times. Other factors therefore appear to have greater influence on brain concentrations of this neurotransmitter during the feeding of these diets. There is no evidence that catecholamine concentrations are affected by the level of food and hence precursor intake.

ii Effects of age and length of the feeding period

The brain concentrations of the neurotransmitters and metabolites measured also show some increase with the age of the chick and length of the feeding period. While increases in NE, DA, DOPAC, HVA and 5HT concentrations are observed at various times in chicks receiving the control diet, birds fed the diet containing excess lysine alone show a rise in the brain concentrations of NE and DA only. In birds fed the diet supplemented only with arginine, the brain concentration of NE alone rises significantly with age, while those consuming the diet incorporating both excess lysine and additional arginine show increases in the brain concentrations of none of the measured compounds. Such data would appear to indicate that lysine and arginine each act in some way to prevent increases in the brain concentrations of the relevant

neurotransmitters and metabolites occurring with age of the chick and length of the feeding period. The causes of the observed variations in neurotransmitter concentrations with time are not apparent.

It thus appears that the interactions between dietary lysine and arginine do have some effect on the brain neurotransmitter concentrations of chicks. Differences tend to be greatest between birds fed the diet containing excess lysine alone and those fed that incorporating both lysine and additional arginine. Where an effect is seen, excess lysine tends to reduce concentrations of the measured compounds and arginine to increase these somewhat. The reason for this action where it is observed, is unclear, but cannot be attributed entirely to the differences in food intake of birds fed the different diets. Although it might be suggested that excessive lysine could perhaps competitively interfere with the brain uptake of the neurotransmitter precursors, a role for supplemental arginine in removing such competition is not apparent. It should perhaps be noted here that the concentrations and proportions of the competing BCAA remained constant in relation to each other and tryptophan, tyrosine and phenylalanine in all the diets fed, and no effect can be attributed to alterations in their intake.

It has been mentioned that the observed differences in the brain concentrations of NE and DA at least, in birds fed the different diets, cannot in this study be attributed to differences in their intake of the amino acid precursors. While it is not totally inconceivable that some difference in the amino acid balance of the diets might bring about variations in neurotransmitter concentrations by an unknown mechanism not involving alterations in the availability of their precursors due to altered competition for brain uptake, it must be remembered that after

eight days of feeding the diets, when significant differences in intake of the various diets were very apparent, no significant differences in the brain concentrations of the measured neurotransmitters were observed. There is thus no evidence here that the brain concentrations of these compounds may influence food intake.

4.4 Experiment 3. The effect of supplementary glycine and arginine on methionine toxicity

a. Dietary nitrogen content (Table 14)

As in the previous investigation, the growth and food intake of birds fed the experimental diets did not appear to be attributable to the differences in dietary nitrogen content, the diet containing excess methionine having a greater nitrogen content than the control diet but supporting poorer growth and food intake.

b. Growth and food intake (Figs. 16 and 17)

Very little evidence has been provided by this investigation for the alleviation by glycine or arginine of the adverse effects of excess methionine on chick growth and food intake. This contrasts somewhat with the work of Benevenga and Harper (1967), who had indicated that in the rat, the growth- and food intake-depressing effects of methionine added to the diet at a concentration of 30g/kg were partially alleviated by a glycine supplement of 15g/kg. In the chick, it was reported that the adverse effect of methionine added at 8g/kg was more than overcome by a glycine supplement of 10g/kg (Waterhouse and Scott, 1961) while some alleviation of the effect of methionine added to the diet at a concentration of 10g/kg was also obtained by supplementation with arginine at 3g/kg (Smith, 1969). It is therefore possible that greater supplements of these two amino acids might have resulted in an improvement in growth or intake reaching

significance, although none was obtained in the current study. Nevertheless. the addition of glycine in the absence of supplemental arginine appeared to be of some benefit.

c. Neurotransmitter concentrations (Table 16)

i.Effects of diet and food intake

The increase in the brain concentration of DOPAC in chicks fed the diet supplemented with both methionine and glycine for five days in comparison with those fed all other diets may indicate an increased turnover of DA, no differences in the brain concentration of this neurotransmitter being apparent. Since chicks fed the various diets at this time showed no significant differences in their brain concentrations of NE and DA, and there was no correlation of the concentration of either of these compounds with the level of intake of food, protein or the catecholamine precursors, it does not appear that the concentration of either neurotransmitter was affected by the differing levels of food intake of chicks fed the various diets.

In comparison with birds fed certain of the other diets, the reduced brain concentrations of 5HT in chicks fed diets containing excess methionine and supplemental glycine alone or with arginine, might be taken to be a result of their reduced tryptophan intake. However, chicks fed the diet incorporating excess methionine alone had a greater brain concentration of 5HT, yet a level of food intake which was initially less and never greater than that birds fed a concomitant supplement of glycine. This, together with the lack of correlation of brain concentrations of 5HT with the level of food, protein or tryptophan ingested, indicates that differences in food intake are not a cause of the differences in brain 5HT concentrations.

After nine days of feeding the experimental diets, the increased brain concentration of HVA of chicks consuming excess methionine in the presence of supplementary glycine alone or with additional arginine again indicates a possible increase in turnover of DA. It is noticeable that at this point of the feeding period, when differences in food intake-at least between chicks fed the control diet and those receiving the others-are very apparent, no other differences in brain neurotransmitter concentrations were observable. Again, there are no indications that the brain concentrations of NE, DA or 5HT are influenced by the quantity of food, protein or amino acid precursors consumed.

At the end of the experimental period, the lowered brain DA concentrations of chicks fed the diet containing excess methionine and supplemented with arginine+glycine, relative to that of those fed either of the other diets containing excess methionine, seems to indicate that an increase in DA breakdown or fall in its synthesis is occurring. The increase in brain DOPAC concentrations of birds fed the diet incorporating excess methionine with additional glycine and arginine relative to that of those receiving only excess methionine, supports the possibility of an increased breakdown of DA in chicks fed the former diet.

Excess dietary methionine thus affects neurotransmitter metabolism in the brain of the chick to only a small extent. Few significant differences in actual concentrations of the neurotransmitters or metabolites measured are apparent, although there are indications of an increased turnover of DA in the brains of chicks fed diets containing excessive methionine and supplemented with both glycine and arginine. There is no evidence of the brain concentrations of NE, DA or 5HT being

affected by or related to the amounts of food, protein, or the precursor amino acids consumed. While methionine is reported to be a possible competitor with the other LNAA for uptake into the brain (Olendorf, 1971; Pardridge and Olendorf, 1975), even at the high concentrations included in the diet here, no evidence of such an effect and a consequent reduction in neurotransmitter concentrations is apparent.

ii. Effects of age and length of the feeding period

Certain changes in the concentrations of particular neurotransmitters and metabolites with age were apparent during the feeding of the diets of the previous investigation. For chicks fed any one of the diets of this study, the absence of any significant change in the brain concentrations of any of the measured compounds with age and length of the feeding period suggests that such effects may depend upon the composition of the diet itself. As other workers have indicated that the blood-brain barrier of the chick continues to develop during the first four weeks of life and that the brain concentrations of certain amino acids fluctuate over this period (Levi and Morisi, 1971; Purdy and Bondy, 1976), changes in neurotransmitter concentrations in the brain due to alterations in the availability of the amino acid precursors might have been expected to occur with age. No evidence of such an effect is seen here.

4.5 Experiment 4. The induction of a dietary tryptophan imbalance

a. Dietary nitrogen content (Table 14)

It does not appear that the differences in growth and food intake of chicks consuming each of the diets fed during this experiment can be attributed to the different dietary nitrogen contents, no consistent variation of chick growth and food intake with dietary nitrogen content being apparent.

b. Growth and food intake (Figs. 18 and 19)

A dietary amino acid imbalance with respect to tryptophan does not appear to have been produced in this investigation, addition of the indispensable amino acid mixture to the control diet having no effect, although supplementation with tryptophan did improve chick performance somewhat. The poor growth supported by the basal diet even when additional tryptophan was incorporated, was almost certainly due to the type of gelatin employed when constituting the experimental diets. This was less costly than that employed in all other feeding experiments, but had a higher bloom value, this being an index of the strength of the gels formed by the gelatin. Despite the level of incorporation being fairly low, diets containing this gelatin were subsequently found to become sticky and adhere to the chicks' beaks. All beaks were cleared at each time of weighing but feeding difficulties were obviously experienced. It is thus likely that the growth of chicks fed the control diet in this investigation was not sufficiently good for the administration of an imbalanced amino acid mixture to have any further observable effect on chick performance.

It is also possible that the initial dietary tryptophan concentration in the control diet was perhaps too low for effects of a further deficiency induced by addition of the indispensable amino acid mixture to be observed. However if this had been the case, it might have been expected that the diet containing both additional tryptophan and the imbalanced mixture of amino acids would have supported poorer growth and intake than that to which only tryptophan had been added. This however was not observed. A further possibility is that the amino acid mixture employed to produce an imbalance -based on that used by Sanahuja and Harper (1963b)- was not large enough to produce a

sufficiently great relative deficiency of tryptophan. The fact that the diets employed were not niacin-deficient may also have been a contributory factor, since reported imbalances with respect to tryptophan tend to be produced by utilising basal diets deficient in this vitamin. Niacin is produced from tryptophan and may partially compensate for its absence (Koeppe and Henderson, 1954; Morrison and Harper, 1960; Wilson, Wortham, Benton and Henderson, 1962).

c. Neurotransmitter concentrations (Table 17)

i. Effects of diet and food intake

The increased brain concentration of HVA apparent after five days of feeding in chicks fed diets supplemented with tryptophan compared with those fed the control diet, and at the end of the experimental period in comparison with either of the diets not supplemented with this amino acid, is indicative of an increased turnover of DA in the brains of birds fed tryptophan-supplemented diets. No comparable change in the concentration of DOPAC is observed to support this however. Greater concentrations of 5HT and 5HIAA in the brains of chicks fed tryptophan-supplemented diets for five days are consistent with the synthesis of these compounds being dependent on the availability of tryptophan to the brain (Fernstrom and Wurtman, 1971b; Fernstrom, Larin and Wurtman, 1973). This is also supported by the similar trends in brain concentrations of 5HT after nine days of feeding, and in the concentrations of both 5HT and 5HIAA at the end of the experiment. Throughout the feeding period, the observed positive correlation of chick brain concentrations of 5HT with tryptophan intake would also support the existence of such a dependence of the neurotransmitter concentration on the availability of its precursor.

It should be noted that after five days of feeding, the brain concentration of 5HIAA in chicks receiving the diet incorporating both the indispensable amino acid mixture and additional tryptophan was less than that of birds receiving the tryptophan supplement alone. It is at this point that the possibility of an effect of the indispensable amino acid mixture might be considered. Increased competition with tryptophan for uptake into the brain, provided by the LNAA of the added mixture (Olendorf and Szabo, 1976), could reduce the availability of tryptophan for 5HT synthesis. In order that brain concentrations of 5HT might be maintained, the breakdown of this neurotransmitter might be expected to fall, resulting in a decrease in the brain concentration of 5HIAA.

While such an effect is consistent with the result obtained here, it would then be expected that birds fed the amino acid mixture in the absence of additional tryptophan would have lower brain concentrations of 5HIAA or 5HT than those fed the unsupplemented control diet. This was not in fact observed. This may possibly be due to the extremely low tryptophan concentration of the control diet resulting in minimum brain concentrations of these compounds already being acheived, an absence of competition between the LNAA for brain uptake, or some other mechanism which remains unclear. In addition, at the end of the experiment, the brain concentration of 5HT in chicks fed the diet containing both the indispensable amino acid mixture and additional tryptophan was not significantly different from that of those receiving the diet incorporating only the amino acid mixture. It might have been have been expected that birds fed the former diet would have had the higher brain concentration of 5HT, due to the actual increase in intake of tryptophan or the increase in the ratio of tryptophan intake to that

of its proposed competitors for brain uptake. The absence of such an effect is somewhat surprising because of the positive correlation of 5HT concentrations with the level of tryptophan intake, but it is possible that the presence of the LNAA mixture has provided sufficient competition to prevent any increase in availability of tryptophan to the brain for 5HT synthesis (Fernatrom, Larin and Wurtman, 1973).

Thus the brain concentration of 5HT in chicks fed a tryptophan-deficient diet may be increased by supplementation with tryptophan. Some influence of other added LNAA is suggested, although this may only affect the 5HT concentration when it is above a particular minimum. The positive correlations of brain concentrations of 5HT with the quantities of food, and protein ingested must be considered in the light of the knowledge that birds having the greatest food and protein intake are those consuming the diets containing the greatest amounts of tryptophan. These observed correlations are likely to be a product of the changes in tryptophan intake.

ii. Effect of age and length of feeding period

The occurrence of little change in the brain concentrations of the measured neurotransmitters and metabolites with chick age and the length of the feeding period again indicates, as in the previous investigation, that the metabolism of these compounds is altered to a very small extent in the first three weeks of the life of the chick.

4.6 Experiment 5. Amino acid imbalance and responses to tryptophan supplementation

a.Dietary nitrogen and AME(N) content (Table 18)

The variations in the dietary content of neither nitrogen nor AME(N) were consistent with the observed differences in growth and food intake of birds fed the different diets. While it might have been

argued that the reduction in dietary AME(N) with increasing tryptophan content in the diluted series of diets was responsible for the corresponding increases in chick food intake, the diluted diet containing 0.63 of the chick's tryptophan requirement had a greater AME(N) and also supported a greater chick growth than the supplemented diet containing the minimum amount of tryptophan. It therefore is unlikely that the determined variations in dietary AME(N) content make a major contribution to the observed differences in growth and food intake responses of chicks fed the different diets.

b. Growth and food intake (Figs. 20 and 21)

The growth and food intake of birds fed the experimental diets tends to increase with tryptophan supplementation. For birds receiving diets containing the same amount of tryptophan, those consuming the diluted diet have a somewhat greater food intake, but this does not appear to be attributable to differences in dietary AME(N) content. While the two methods employed for determination of amino acid requirements here support slightly different patterns of food intake, the growth responses and patterns of daily weight gain with daily intake of tryptophan are essentially the same. Each method is therefore equally valid for the estimation of amino acid requirements of the young broiler chick, as indicated by D'Mello (1982). In each case however, the daily gain does not appear to have levelled off completely at the higher levels of tryptophan intake and it is possible that a slight increase in dietary tryptophan content might still have improved the growth response. It thus appears that the currently estimated level of requirement of tryptophan (Agricultural Research Council, 1975) is slightly lower than that needed for optimal chick growth, and that a

further increase in dietary tryptophan content might still have beneficial effects.

c. Neurotransmitter concentrations (Table 19)

The lower brain concentration of E in chicks fed the supplemented diet containing tryptophan at 0.78 of its required concentration in comparison with that of those fed the other diets of this series, might suggest that as the dietary tryptophan concentration is increased, availability of the catecholamine precursor in the brain begins to fall. Chicks fed the diets of the diluted series formulated to contain 0.78 or 0.63 of the required concentration of tryptophan however, showed lower brain E concentrations than those fed the diluted diet adequate in its tryptophan content. These observations appear to conflict with any competitive effects which might be expected between the LNAA for uptake across the blood-brain barrier by a common transport mechanism (Olendorf and Szabo, 1976), as in the diets of the diluted series the relative proportions of the amino acids remain constant. Thus diets containing a higher concentration of tryptophan would also have a proportionately greater concentration of tyrosine and phenylalanine and should therefore cause no change in the brain concentrations of the catecholamines or 5HT. The reduced brain concentration of DA in chicks receiving the diluted diet containing 0.78 of the required amount of tryptophan compared with those receiving 0.48 of the tryptophan requirement by the method of graded supplementation is therefore also surprising, but the lack of any reduction in NE or DA concentrations in the brains of birds fed the diluted diet adequate in its tryptophan content compared with those fed any of the other diets is more understandable.

The increased brain concentrations of 5HT and 5HIAA in chicks fed the diluted diet designed to be adequate in its tryptophan content compared with that measured in birds receiving the other diets, implies that the brain concentration of this neurotransmitter is indeed influenced by the availability of its amino acid precursor, as proposed by Fernstrom and Wurtman (1971b). The positive correlation of chick brain concentrations of 5HT with the amount of tryptophan consumed is fairly weak, but would support this suggestion.

Comparison of the groups of chicks fed the diluted diets with each other has shown that the brain concentration of 5HT is higher in chicks fed that adequate in its tryptophan content than in those receiving lesser amounts. As the ratio of all the amino acid concentrations is constant, possible effects due to changes in competition are ruled out, and the increase appears to result merely from an increase in actual tryptophan intake. However, diets formulated by the different methods to contain the same amount of tryptophan have quite different ratios of this amino acid to the total dietary concentration of the LNAA. As no significant difference was observed in brain neurotransmitter concentrations of chicks fed diets containing identical amounts of tryptophan but formulated by the different methods, it is apparent that in this feeding experiment no effect on neurotransmitter concentrations has been produced by an alteration of the relative proportions of dietary LNAA. Thus there is no evidence here of the existence of competition between the LNAA for uptake into the brain. This could possibly be attributed to the suggested immaturity of the blood-brain barrier in chicks of this age (Levi and Morisi, 1971; Purdy and Bondy, 1976).

4.7 Experiment 6. Zinc deficiency in the chick and an amino acid imbalance with respect to tyrosine and phenylalanine

a. Dietary nitrogen and AME(N) content (Table 20)

Differences observed in the nitrogen contents of the diets fed are unlikely to have been a major factor in causing the observed differences in growth and intake of the chicks to which they were fed, there being no obvious parallel between the dietary nitrogen content and the levels of chick growth or food intake. Similarly, differences in the dietary AME(N) content show no consistent trend with either the food intake or growth of birds fed the various diets.

b. Growth and food intake (Figs. 23 and 24)

For either the zinc-deficient or the zinc-adequate group of diets, addition of the mixture of indispensable amino acids depresses chick growth and food intake in comparison with the control diet in the presence or absence of zinc, despite the increased crude protein content and identical concentrationof tyrosine and phenylalanine . These effects are alleviated by supplements of phenylalanine and it is therefore apparent that an imbalance with respect to tyrosine and phenylalanine was acheived upon addition of the amino acid mixture, a relative deficiency of these amino acids being aggravated and supplementary phenylalanine improving growth and intake by restoring the amino acid balance.

In most cases zinc deficiency has no significant effect, or indeed reduces the weight gain and food intake of chicks relative to these measurements made on chicks fed the comparable zinc-adequate diet. However in the case of the imbalanced diets greater growth and food intake is obtained, at least initially, for chicks consuming the zinc-deficient form. Thus zinc deficiency appears to have a beneficial effect

on the performance of chicks fed a diet which is relatively severely deficient in tyrosine and phenylalanine. This contrasts somewhat with the results of Reeves and O'Dell (1984) who indicated that while the lowering of the dietary concentration of these amino acids aided the growth and food intake of rats fed a zinc-deficient diet, lowering zinc concentrations was detrimental to the performance of rats fed a diet which was low in its tyrosine and phenylalanine content. It should be noted however that in their investigation, intake of the diet which was low in these amino acids but adequate in zinc was not reduced relative to that of a fully-supplemented diet, while the creation of a relative deficiency by the imbalance described here did have an adverse effect on food intake. In both cases when the diet is well-balanced as regards amino acid levels, zinc deficiency reduces the weight gain and food intake responses obtained.

c. Neurotransmitter concentrations (Table 21)

The lack of effect of this amino acid imbalance on brain catecholamine levels could perhaps be argued to be due to the proposed absence of a blood-brain barrier in the young chick (Levi and Morisi, 1971; Purdy and Bondy, 1976), as brain catecholamine levels are unchanged unless the absolute dietary concentrations of tyrosine and phenylalanine, rather than merely their concentrations relative to the other LNAA, are altered. Alternatively, a relative reduction in the dietary concentrations of the catecholamine precursors might have been insufficient to affect the brain pools of these amino acids and hence brain concentrations of NE and DA. It is also conceivable that the apparent absence of blood-brain barrier competitive effects in the case of tyrosine and phenylalanine could be caused by their being transported into the chick brain by a system which was different from that in the

rat and did not also transport other LNAA. It is perhaps more likely however that chick brain tyrosine hydroxylase has an affinity for tyrosine sufficient to protect the catecholamine synthetic process from some reduction in precursor concentrations as might have been acheived by this method.

A further possibility is that the brain concentrations of DA and NE in chicks fed the low-protein control diet are already at a minimum due to the low dietary concentrations of tyrosine and phenylalanine, and therefore addition of the imbalancing amino acid mixture has no further effect. Increasing phenylalanine administration would still enable increased synthesis of the catecholamines, as was implied here.

The absence of an effect of addition of the imbalancing amino acid mixture to the control diet on brain concentrations of 5HT and 5HIAA should perhaps also be considered. In adding this mixture the dietary concentration of the 5HT precursor tryptophan was increased and concentrations of leucine, isoleucine and valine were increased correspondingly so that the same ratio of these amino acids to tryptophan was maintained. However as tyrosine and phenylalanine concentrations in the imbalanced diet remained unaltered, the dietary ratio of tryptophan to the total concentrations of two amino acids rose. Subsequent supplementations with phenylalanine gradually restored this ratio to that of the control diet.

Assuming that competitive transport into the brain of these six large neutral amino acids occurred in accord with the reports of Olendorf and Szabo, 1976), then it might have been expected that in chicks fed the imbalanced diets an increased amount of tryptophan would have been taken into the brain, leading to higher concentrations of 5HT

and possibly 5HIAA. If during the experimental period there was in fact no competition for brain uptake across a blood-brain barrier in the chick, then the increased dietary concentration of tryptophan alone would still have been expected to raise brain concentrations of 5HT. Such an effect was not apparent here however. It appears that some sort of competitive effects may exist to influence the uptake of tryptophan into the brain, the accompanying rise in dietary branched-chain amino acids possibly providing sufficient competition to prevent the rise in dietary tryptophan causing a corresponding increase in the brain concentrations of this amino acid and hence 5HT.

Zinc deficiency appears to reduce the brain concentrations of the catecholamines, as shown by the greater brain concentrations of NE and DA in chicks receiving the zinc-adequate rather than the zinc-supplemented imbalanced diets. In addition, the brain DA concentration of chicks fed the zinc-supplemented diet containing the imbalancing amino acid mixture and a phenylalanine supplement of 5g/kg, and NE, DA and DOPAC concentrations of chicks fed the imbalanced diet supplemented with phenylalanine at 10g/kg, were greater than in those consuming the comparable diets lacking zinc.

While zinc deficiency had no significant effect on the brain concentrations of 5HT, some effect on 5HIAA concentrations was apparent, these being reduced in chicks fed the zinc-deficient imbalanced diet alone or with phenylalanine at 5g/kg, compared with those receiving a similar diet which was supplemented with zinc. This may be an indication of a reduced turnover of 5HT, a reduction in 5HT breakdown occurring in parallel with a fall in its synthesis.

Reeves and O'Dell (1984) found no significant effect of feeding zinc-deficient diets on the brain concentrations of the

catecholamines. They did however report reduced brain levels of tyrosine in rats fed diets which were deficient in zinc, which might account for the decreases in brain catecholamine concentrations observed during this investigation in the chick. The increased concentration of NE in the anterior hypothalamus of rats during zinc deficiency as reported by Wallwork, Botnen and Sandstead (1982) is not necessarily in conflict with these data as it is possible that such an increase was a localised effect, whole brain levels not having been measured in this case. The different effects could also have been caused by dietary differences or the fact that zinc deficiency was overcome in these latter workers' experiments by addition of zinc acetate to the drinking water rather than by modification of the diet. It is conceivable that such a remedy for zinc deficiency might affect food and water intake-regulating mechanisms in a way other than that by which effects were brought about in the current investigation. This might then lead to differences in the measured catecholamine levels, whether directly as part of the mechanism itself or by a more indirect route.

Wallwork *et. al.* (1982) suggested that the aldehyde dehydrogenase enzyme which acted in conjunction with monoamine oxidase to bring about neurotransmitter breakdown required zinc for its activity in a similar manner to other NAD^+ and $NADP^+$-utilising dehydrogenases, and that the increase in catecholamine concentrations caused by zinc deficiency might be due to a reduction in the activity of this enzyme. However, a similar argument could be used to account for the reduction in brain catecholamine concentrations seen in this investigation. The hydroxylase enzymes involved in the synthesis of NE, DA and 5HT require a tetrahydrobiopterin prosthetic group which, after oxidation to dihydrobiopterin during the activity of the enzyme, is reduced once more

by the action of an $NADP^+$ -requiring dihydrobiopterin reductase (Bender, 1978). This is equally likely to be a zinc-containing enzyme and a deficiency of zinc would then result in a loss of activity of the brain's hydroxylase enzymes which might then be followed by a fall in the concentrations of the measured neurotransmitters.

The lack of correlation of brain concentrations of NE, DA or 5HT with the levels of intake of food, protein, AME(N) or their amino acid precursors would appear to support the importance of the relative amino acid balance of the diet, rather than its actual amino acid content, in determining the neurotransmitter concentrations of the brain.

There is little evidence to suggest that the observed changes in the concentrations of the neurotransmitters and their metabolites is infuential in determining the levels of food intake and growth of the chicks fed the experimental diets. It might be argued that a reduction in brain catecholamine concentrations caused by a deficiency of zinc could be beneficial in alleviating the adverse effects of this imbalance on chick growth and food intake while having no effect on the performance of chicks fed more balanced diets. However it should be noted that the highest brain NE levels are found in those birds showing the best performance overall.

4.8 Experiment 7. Effect of ephedrine on a dietary tyrosine+phenylalanine imbalance

a. Dietary nitrogen and AME(N) content (Table 22)

The variation in nitrogen content of the diets fed during this investigation is much as for comparable diets of the previous experiment and differences in the level of weight gain and food intake supported by the various diets are not considered attributable to the differences in their dietary nitrogen content. Comparison of the

determined AME(N) contents of the diets with the growth and food intake supported by each also indicates that the differences in dietary AME(N) are not ultimately responsible for the measured differences performance of chicks consuming them.

b. Growth and food intake (Figs. 25 and 26)

The growth and food intake of chicks fed diets identical to those employed as the zinc-adequate series of the previous experiment showed some variation from the comparable data obtained in that case. The failure of chicks fed the ephedrine-lacking imbalanced diet to show a net weight loss as was observed for those receiving the identical diet in the previous investigation can only be attributed to variations in the individual chicks employed, since when experimental diets were first presented the mean weight of chicks in each investigation was similar.

In all cases addition of ephedrine to the diet tended to reduce the weight gain and food intake of chicks fed this rather than the comparable diet lacking the drug. It appears that adaptation to the presence of ephedrine occured within the experimental period when the diet into which it was incorporated was imbalanced. Only in this case did the differences in gain and intake between chicks fed diets with and without the thermogenic drug reach a point after which they were not significant. However it should be borne in mind that as the variation in response of chicks fed the imbalanced diets was rather greater than that of those fed the others, the comparison of two such diets might conceivably result in no significant difference being obtained between the two at some point.

c. Neurotransmitter concentrations (Table 23)

The greater brain concentration of NE in chicks receiving the ephedrine-lacking diet incorporating the imbalancing amino acid mixture and additional phenylalanine compared with those fed the control diet, seems to indicate that the concentration of dietary phenylalanine does here influence brain concentrations of NE. There is however, no general correlation of brain NE concentrations with the quantities of tyrosine+phenylalanine consumed. The lack of effect of addition of the indispensable amino acid mixture on brain NE concentrations suggests that increasing the dietary concentration of LNAA other than tyrosine or phenylalanine does not decrease the brain uptake of the latter amino acids by competitively inhibiting their uptake (Olendorf and Szabo, 1976). No evidence is therefore apparent here for competition between the amino acid precursors and other LNAA for uptake into the brain. An increase in brain catecholamine concentrations on increasing dietary phenylalanine however, would be supported by the greater brain concentration of E in chicks fed the phenylalanine-supplemented diet containing the indispensable amino acid mixture and lacking ephedrine compared with those fed the drug-supplemented control diet.

In contrast to the above observations, the brain DA concentration of chicks fed the totally unsupplemented control diet was greater than that of those fed any of the other diets, suggesting that the concentration of this catecholamine was not readily affected by changes in the phenylalanine content of the diet. This would be supported by the lack of correlation of brain concentrations of DA with the amounts of tyrosine+phenylalanine ingested by the chick. The lower DA concentration in the brains of chicks fed the imbalanced diets lacking supplemental phenylalanine would be consistent with the

functioning of a blood-brain barrier and competition between the LNAA for brain uptake, but the failure of additional phenylalanine to restore NE concentrations to control values does not support the existence of any influence of dietary phenylalanine concentrations on the brain concentration of DA, with or without such competition.

The lack of effect of feeding the different diets on the brain concentration of 5HT raises two main points. Firstly it is apparent that at least within the dietary range here, the concentration of tryptophan in the diet has little effect on the brain 5HT concentration, since this amino acid is at a much lower concentration in the control diet than in any of the others and there is also no correlation of brain 5HT concentrations with the quantity of tryptophan consumed. Secondly, the failure of additional phenylalanine to reduce brain 5HT confirms that in this investigation no evidence is available of competition between the LNAA for brain uptake.

The reason for the greater brain concentration of 5HIAA in chicks receiving the diet incorporating both the indispensable amino acid mixture and ephedrine than in those fed any of the other diets is not immediately clear. This possible increase in turnover of 5HT could be caused by the addition of either the drug or the indispensable amino acid mixture, but as the effect is seen only for birds fed this diet, evidence for either possibility is not great.

As birds fed the diet incorporating the imbalancing amino acid mixture and additional phenylalanine in the absence of ephedrine were also shown to have a higher brain concentration of NE than those fed any of the the drug-supplemented diets, it appears that ephedrine tends to reduce the brain concentration of this and therefore possibly other catecholamines. As the drug fenfluramine has been shown to

increase the release of 5HT and so deplete its concentration in the brain (Trulson and Jacobs, 1976; Fuller, Snoddy and Hemrick, 1978), so it might be expected that ephedrine, which stimulates the release of NE into the synaptic cleft (Kruk and Pycock, 1979), will similarly reduce the brain concentration of NE. The greater brain concentration of E in chicks fed the drug-lacking diet containing the indispensable amino acid mixture and supplemented with phenylalanine compared with those fed the drug-supplemented control diet could also be partially due to such a catecholamine-depressing effect of ephedrine, in addition to a possible phenylalanine-caused increase in this catecholamine.

As in the previous investigation, the absence of any correlation of chick brain concentrations of NE, DA or 5HT with the levels of intake of food, protein, AME(N) or the relevant amino acid precursors indicates that the observed effects on these compounds are unlikely to be due to alterations in the actual intake of any of these.

4.8 Experiment 8. The effects of increased incorporation of balanced and imbalanced proteins into the diet

a. Dietary nitrogen and AME(N) content (Table 24)

The increase in dietary nitrogen content with increasing incorporation of protein into the diet is the most probable cause of the fall in food intake with increasing incorporation of protein. The tendency of diets incorporating gelatin to have a higher nitrogen content than those containing casein and designed to contain identical amounts of nitrogen might also be a reason for their supporting somewhat lower levels of growth and food intake. However, it appears unlikely that the lower AME(N) contents of gelatin-containing diets is a factor in their reduced intake.

b. Growth and Food Intake (Figs. 27 and 28)

When the casein-containing diets are considered, it appears that the intake of diets containing increased levels of nitrogen is initially reduced, a period of adaptation being required before it is restored to control levels. Consumption of diets containing the lower concentrations of casein is fully restored within the experimental period but no obvious adaptation to the diet into which the greatest amount of casein is incorporated is seen during this time. The weight gain of chicks is unaffected by the reduced intake of diets incorporating the lower levels of casein supplementation, the increased nitrogen content and possibly also the slight rise in AME(N) presumably compensating for any fall in intake. However the depression in intake occurring in chicks consuming the greatest amount of casein appears to be sufficiently severe as to prevent or at least delay any possible compensatory effects of these increases.

With regard to the gelatin-supplemented diets, the lowest level of gelatin supplementation leaves growth unaffected while intake undergoes a period of adaptation. At a gelatin concentration equivalent to a nitrogen supplement of 29g/kg growth is depressed in addition to intake, the latter being restored to control levels within the experimental period. This indicates that the observed depressions in growth and intake were not due to difficulty in consumption of the feed because of the relatively large amount of gelatin employed. The use of gelatin of a low 'bloom' value had thus been somewhat successful in preventing possible feeding difficulties. In the case of chicks receiving the greatest quantity of gelatin however this was not the case, the diet becoming sticky, adhering to the birds' beaks and making food consumption difficult. The effects observed on feeding this diet must

therefore be set aside since factors are involved other than those under investigation.

Thus it appears that adaptation to the intake of a high-protein diet does indeed occur in the chick, food intake initially being depressed and subsequently returning to control levels unless the protein content of the diet is very high. This is comparable to the effects reported by Peng, Meliza, Vavich and Kemmerer (1974) with rats. The effect seems to occur whether dietary AME(N) increases with increasing protein and hence nitrogen content -as in the case of casein-containing diets- or falls as protein levels rise -as is seen when gelatin is incorporated. When the effect of the diet is such that the depression in intake is sufficiently severe, the weight gain of the chicks falls also.

It appears in addition that if the effects of incorporating casein and gelatin at a nitrogen concentration of 29g/kg are compared, then while in each case an initial depression in food intake is later restored, in the case of the gelatin-containing diet a reduction in weight gain is also seen. The difference does not appear to be due to a difference in the dietary nitrogen content actually determined but may be due to the lower AME(N) content of the gelatin-containing diet. The increased dietary nitrogen content appears to be such that an increase in food intake in response to the reduction in dietary AME(N) is prevented. Anderson (1979) suggested that unless the distortions in plasma and brain amino acid concentrations caused by ingestion of a diet were very severe, the regulation of energy intake took priority over that of protein. It therefore appears that the feeding of high concentrations of an imbalanced protein such as gelatin may cause a greater distortion in the plasma and brain amino acid patterns and have

a more detrimental effect on chick performance than an identical amount (with regard to nitrogen content) of a balanced protein such as casein.

c. Neurotransmitter concentrations (Table 25)

It appears that increasing the protein, and hence nitrogen content of the diet tends to decrease the brain concentration of NE. Chicks fed diets incorporating gelatin at the two lower nitrogen concentrations had a reduced brain concentration of NE compared with those fed the control diet, while birds fed the diet containing casein equivalent to a dietary nitrogen concentration of 29g/kg showed a brain NE concentration below that of those fed the control diet or that supplemented with the lower concentration of casein. These data indicate that the reduction in brain NE concentrations may occur at lower concentrations of the imbalanced protein than of the more well-balanced casein. Of the birds fed the three diets mentioned previously, only chicks fed the diet containing gelatin equivalent to a nitrogen content of 29g/kg had a weight gain and food intake less than that of those fed the control diet. The intake of tyrosine and tryptophan however, increased with increasing supplementation of the diet with either gelatin or casein, and it is therefore apparent that the differences in brain NE concentration are not due to differential consumption of its precursors. The lack of correlation of brain NE concentrations with the amount of tyrosine+phenylalanine consumed would confirm this. In addition, birds consuming diets containing supplements of either gelatin or casein greater than those mentioned showed no difference in brain NE concentration compared with those fed the control diet, despite differences in food and hence tyrosine and phenylalanine intake.

The increased brain concentration of E in chicks fed the diet incorporating the highest concentration of casein compared with

that of birds fed gelatin at any level of supplementation might have been attributed to the greater intake of catecholamine precursor by chicks fed this diet. The observation that birds fed the greatest concentration of gelatin had a higher brain E concentration than chicks fed an amount of casein equivalent to a dietary nitrogen content of 16g/kg, would be consistent with the greater intake of tyrosine and phenylalanine by chicks fed the former diet, despite the lower food intake. However, if such observed changes were indeed a result of altered intake of the amino acids mentioned, birds fed the control diet should have shown lower concentrations of E than those fed any other diets. This was not in fact seen. The reason for the described increases in E and also in the brain concentration of DA in chicks fed the diet incorporating the greatest concentration of gelatin compared with those receiving casein equivalent to a dietary nitrogen concentration of 29g/kg, cannot therefore be readily explained. The lack of correlation of brain DA concentrations with the quantities of tyrosine and phenylalanine consumed again indicates that differences in the concentration of this neurotransmitter are not due to differences in the intake of its precursor amino acids.

Reasons for the determined variations in the chick brain concentration of 5HT are also not easily obtained. A reduction in brain 5HT concentrations in chicks fed diets containing casein equivalent to a nitrogen content of 29 g/kg or gelatin equivalent to nitrogen contents of 16g/kg and 48g/kg cannot be explained by a decreased intake of tryptophan relative to birds fed each of the other diets, there being no correlation between the brain concentration of 5HT and the amount of tryptophan consumed. It might be suggested that as gelatin is relatively deficient in tryptophan, increasing the concentration of this protein in

the diet would raise the concentrations of the other LNAA with which it is proposed to compete for uptake into the brain (Olendorf and Szabo, 1976) by a greater amount than by which tryptophan itself would be increased. This, assuming such competition occurred, would result in a reduction in brain 5HT concentrations with increasing dietary concentrations of gelatin. Such a phenomenon does not however actually explain the absence of a reduction in brain 5HT concentrations in birds fed the diet containing gelatin at a nitrogen concentration of 29g/kg, nor the observed reduction in 5HT concentrations in the brains of chicks receiving the diet containing casein at this level of incorporation. The greater brain concentration of 5HIAA in chicks fed the highest concentration of gelatin indicates a possible increase in turnover of 5HT in these birds, but does not aid clarification of the processes occurring here.

The few differences in brain concentrations of the measured neurotransmitters in chicks fed the diets of this investigation are difficult to explain. Peters and Harper (1981, 1985) and Fernstrom *et al.* (1985) found no consistent correlation of rat brain concentrations of NE, DA or 5HT with protein intake, although Peters and Harper (1985) reported an inverse correlation of the rat brain concentration of HVA with protein intake. The negative correlation of the chick brain concentration of DA in the current study with the level of food intake is fairly weak, but may be related to this latter observation. The positive correlation of brain 5HT concentrations with AME(N) intake is somewhat stronger and could possibly be linked to reports that the consumption of a carbohydrate diet tended to increase the brain concentration of 5HT in the rat, due to an insulin-produced selective lowering of all LNAA but tryptophan in the plasma enabling the increased

brain uptake of this amino acid (Fernstrom, Larin and Wurtman, 1973). Nevertheless, the carbohydrate content of these diets in relation to their AME(N) content cannot accurately be determined.

No consistent alteration in the concentrations of NE, DA or 5HT with changes in intake of their precursor amino acids is apparent, while the incorporation of the imbalanced protein gelatin rather than the balanced casein has no definite effect attributable to this. No effects consistent with LNAA competition are observable, that is, no reductions in catecholamine or 5HT concentrations occur with increasing supplementation of the diet with gelatin. It would perhaps be of interest to study the effect of incorporation of an alternative imbalanced protein into the diet, which did not produce the difficulties in stickiness experienced with gelatin at its highest incorporated level. However, no consistent trends in neurotransmitter concentrations are seen even at the two lower levels of supplementation with this protein.

4.10 Experiment 9. The leucine-valine antagonism

a. Dietary nitrogen content (Table 14)

While the nitrogen content of the control diet was somewhat below that of the others, the growth and food intake supported by it exceeded that of birds fed the diet incorporating excess leucine. The observed differences in growth and food intake of chicks fed the four experimental diets are therefore unlikely to be attributable to the determined, relatively small differences in their nitrogen content.

b. Growth and food intake (Figs. 29 and 30)

The observed patterns of weight gain and food intake of chicks receiving the experimental diets are as expected, excess leucine causing a depression in both of these measurements which is alleviated

by additional dietary valine. As in other studies (D'Mello and Lewis, 1971), little sign of adaptation to the high concentration of leucine in chicks fed the diet incorporating it in the absence of a valine supplement is apparent.

c. Neurotransmitter concentrations (Table 26)

i. Effects of diet and food intake

The greater brain concentrations of HVA after five days of feeding, in chicks consuming the diet containing only the valine supplement compared with measurements made in birds fed either the control diet or that incorporating both excess leucine and additional valine, appears to indicate an increased turnover of DA in these birds. The lack of correlation of the brain concentrations of NE and DA with either the dietary content of tyrosine+phenylalanine or the ratio of this to the total content of their proposed LNAA competitors for brain uptake appears to indicate that the synthesis of these catecholamines is not dependent on the intake of their precursor amino acids, and there is no evidence of competition between the LNAA for uptake into the brain.

The higher brain 5HIAA concentration in chicks consuming the diet supplemented only with valine than in those fed any of the others implies a possible increase in the turnover of 5HT in these birds. The lack of correlation of brain concentrations of 5HT with the amount of tryptophan consumed or with the ratio of the dietary tryptophan content to that of its proposed LNAA competitors for brain uptake suggests that the availability of tryptophan is not here of importance in determining the brain concentration of 5HT.

An increase in synthesis or fall in degradation of NE in chicks fed for nine days the diet supplemented only with valine, is

suggested by their higher brain concentration of this catecholamine in comparison with birds receiving any other diet. The increased brain DA concentration of these birds compared with those fed the control diet, together with the greater brain concentration of E in chicks fed either of the valine-supplemented diets in comparison with that of those receiving the control diet or that containing excess leucine alone, would also be consistent with additional valine tending to raise brain catecholamine concentrations. The lack of significant difference in brain concentrations of DOPAC implies that the rise in DA concentrations at least might be due to increased synthesis rather than a fall in degradation, while the lower brain concentration of HVA in chicks fed excess leucine alone compared with that of those fed the control diet suggests the possibility of reduced turnover of DA here. The increase in brain catecholamines in chicks fed the valine-supplemented diet is not consistent with the proposed competition of valine, as one of the LNAA, with the catecholamine precursors for brain uptake. However, the lack of correlation of brain concentrations of either DA or NE with the quantities of tyrosine+phenylalanine ingested, or the ratio of the dietary content of these amino acids to that of the LNAA proposed to compete with them for brain uptake, indicates that under the conditions of this experiment the brain concentrations of these neurotransmitters are apparently independent of the availability of their precursors in the diet.

The reduced brain concentration of 5HT in chicks fed the diet incorporating excess leucine alone for nine days, compared with that of those fed the diet supplemented only with valine is consistent with the reduction in the brain concentration of 5HT in the rat after

fourteen days of consuming excess dietary leucine as reported by Krishnaswamy and Raghuram (1972). A similar fall in the brain concentration of 5HIAA in these birds below that of those fed the control diet or that supplemented only with valine suggests that synthesis of 5HT is reduced, rather than its breakdown increased.

The results of Krishnaswamy and Raghuram (1972) were obtained during the pairfeeding of rats fed the control diet with those receiving excess leucine and indicate that the reduced brain concentration of 5HT in those fed excess leucine was not due to a difference in the quantity of food ingested. It should be noted however that while the intake of tryptophan would have been equalised by this procedure, that of leucine would not. The possibility of increased dietary leucine causing an increase in total LNAA competition with tryptophan for brain uptake, resulting in a reduced brain concentration of this amino acid and hence 5HT would therefore remain. However, in the current study, the reduction in 5HT synthesis may be attributed to the reduction in tryptophan intake of birds fed the diet containing excess leucine, there being a weak correlation of the brain concentration of 5HT with the level of tryptophan intake. In addition, the fall in 5HT concentrations cannot be said to result from an increase in competition for brain uptake caused by leucine, since there is no correlation of brain 5HT concentrations with the ratio of tryptophan intake to that of its proposed LNAA competitors. This neurotransmitter apparently would therefore be dependent on the level of dietary intake of its precursor as varied by the differences in food intake of the birds consuming the experimental diets.

At the end of the experimental period, the loss of significant differences in the brain concentrations of NE, E, DOPAC, HVA

and 5HT of the birds receiving the various diets are accompanied by a failure of NE, DA or 5HT concentrations to be correlated with the actual intake of their precursor amino acids or the ratios of the dietary content of these precursors to that of their competitors for brain uptake. Alterations in neurotransmitter concentrations, including the maintenance of a higher brain concentration of DA in birds consuming the diet supplemented only with valine compared with those fed the control diet, therefore do not appear to have been due to any changes in the availability to the brain of the precursor amino acids. The higher brain concentration of 5HIAA in chicks fed the diet supplemented only with valine compared with that of those fed the control diet is still apparent and again implies an increase in turnover of 5HT.

The weak correlations of brain concentrations of 5HT with intake of food and protein after nine days of feeding may probably be taken as coincidental with the correlation with tryptophan intake. The total lack of correlation of the brain 5HT concentrations with the ratio of the dietary tryptophan content to that of its proposed LNAA competitors for brain uptake implies that such competitive effects are of little importance in determining the concentration of this compound in the brain, perhaps due to the proposed immaturity of the blood-brain barrier (Levi and Morisi, 1971; Purdy and Bondy, 1976). At no time does the brain concentration of NE or DA appear to be influenced either by the intake of tyrosine+phenylalanine or by the ratio of the dietary content of these amino acids to theat of their proposed LNAA competitors for brain uptake.

ii. Effect of age and length of feeding period

Little effect of chick age and the length of the feeding period on the brain concentrations of the measured neurotransmitters and metabolites is apparent. Some decreases in the concentrations of E, HVA and 5HIAA were observed in birds receiving the control diet or that supplemented only with valine, while a rise in brain E occurred in chicks fed the diet containing excess leucine alone and no effects were seen in those fed the diet concomitantly supplemented with valine. The reasons for and implications of a possible leucine-caused alteration in the patterns of turnover of certain neurotransmitters with time is uncertain.

Information obtained from this experiment, particularly regarding the brain concentrations of 5HT of chicks fed the various diets, contrasts somewhat with that reported by Krishnaswamy and Raghuram (1972). These workers provided data that would be consistent with the operation of a blood-brain barrier in the young rat, raising the concentration of an amino acid competing with tryptophan for brain uptake resulting in a fall in the brain concentration of the neurotransmitter of which tryptophan is the precursor. The apparent lack of a similar effect in the current study seems to imply that in the young chick a blood-brain barrier is not present, or is at a state of development such that the competitive amino acid transport effects investigated here are not yet functioning.

It is also possible that the differences in response obtained were in some way due to the fact that the experiments of Krishnaswamy and Raghuram (1972) were made upon low-protein diets having a protein content of 100g/kg supplied as casein, while these investigations were based upon a control diet with a protein content of

210g/kg. Their control diet was thus reasonably deficient in most indispensable amino acids and addition of excess leucine to this could conceivably produce greater and more lasting effects than an addition made to the current control diet, due to the greater difference between the relative dietary concentrations of each indispensable amino acid and that of leucine.

Certain variations in the brain concentrations of NE, DA, 5HT and the metabolites measured have been obtained by the feeding of diets containing varying concentrations of leucine and valine. Some increase in the catecholamines and 5HT may be caused by the consumption of supplementary valine. The reason for this and other observed effects cannot be explained on the basis of neurotransmitter concentrations being greatly dependent either upon the level of intake of their precursor amino acids or upon the dietary content of these relative to possible competitors for uptake into the brain, although some evidence for 5HT concentrations being dependent on tryptophan intake is provided.

4.11 Experiment 10. Effect of supplementary phenylalanine and tryptophan on the response of the chick to an increased dietary content of the branched-chain amino acids

a. Dietary nitrogen and AME(N) content (Table 27)

Since the control diet had a lower nitrogen content than any of the other diets formulated, yet supported greater levels of growth and food intake, it is unlikely that the dietary nitrogen content was of major importance in bringing about the observed differences in performance of chicks fed the different diets. A similar conclusion may be drawn for the variation in dietary AME(N), differences in both chick growth and food intake being observed where no difference in AME(N) content of the diet fed was apparent.

b. Growth and food intake (Figs 31 and 32)

The lack of significant effect of addition of the first, i.e. lowest level of the BCAA mixture to the basal diet on chick weight gain or food intake, indicates that a balance had been acheived between the relative levels of each of the BCAA and that an adverse effect due to an excess of one of these relative to the other two did not occur.

Benton *et al.* (1956) reported the occurrence of interactions between the BCAA and phenylalanine in the rat, under the appropriate dietary conditions the addition of phenylalanine increasing the requirement of the rat for valine, isoleucine or leucine. This would be consistent with the alleviatory effect of phenylalanine on the dietary excess of the BCAA as seen in the current study. It was suggested by Benton *et al.* (1956) that the effect of phenylalanine might be non-specific, the interactions between the BCAA being due to their structural similarity. It is perhaps possible that the effect of phenylalanine alone and in combination with tryptophan is due to the common mechanism of transport of these and the BCAA into the brain (Olendorf and Szabo, 1976). The addition of phenylalanine and tryptophan would increase the competition between the LNAA for brain uptake and hence reduce entry of the BCAA. This in turn would reduce the distortion in the amino acid pattern of the brain which might previously have brought about the large reduction in food intake of the chick.

It is unfortunate that this experiment had to be terminated after twelve, rather than fourteen days of feeding the experimental diets. This was due to consumption of the control diet being such that its availability to the birds was at this point becoming limiting. Had it been possible to continue feeding for another two days, the beneficial effect of supplementary phenylalanine alone on chicks fed the diet

having maximum supplementation with the BCAA mixture might have reached significance.

c. Neurotransmitter concentrations (Table 28)

The reduction in the brain concentration of DA in chicks fed the diet incorporating the lowest concentration of the BCAA mixture below that of those fed the control diet is accompanied by an increase in the brain HVA concentration and would indicate an increase in breakdown and perhaps also synthesis of DA. Although no similar effect was found for chicks fed the diet incorporating twice this amount of the BCAA mixture, a reduction in the brain concentration of 5HIAA suggests that the turnover of 5HT is reduced in these birds. The reduced brain concentrations of NE, DA 5HT and 5HIAA in chicks receiving the diet containing the maximum concentration of the mixture would support there being a general reduction in the synthesis, or possibly an increase in the breakdown, of the neurotransmitter compounds. Restoration of the brain concentration of NE by supplementation with phenylalanine indicates that the synthesis of this neurotransmitter is here dependent on the dietary concentration of its precursors, the non-significant rise in brain concentrations of 5HT and 5HIAA which accompanied tryptophan supplementation suggesting a possibly similar effect. The return of brain concentrations of NE, DA and 5HT to control values and the significant rise in 5HIAA in the brains of chicks fed the diet incorporating the maximum concentration of the BCAA mixture and concomitantly supplemented with phenylalanine and tryptophan, supports such suggestions.

When considering the changes in both chick growth and brain neurotransmitter concentrations, it does appear that phenylalanine and tryptophan are of greater benefit in alleviating the effects of

excessive dietary BCAA concentrations when the two are fed together rather than singly. The reason for this observation is unclear. The fall in neurotransmitter concentrations caused by the BCAA supplements is consistent with the presence of a functional blood-brain barrier in the young chick, increased amounts of these amino acids causing increased competition with the neurotransmitter precursors for uptake into the brain (Fernstrom, Larin and Wurtman, 1973; Fernstrom, Hirsch and Faller, 1976), and thus reduced synthesis of 5HT, NE and DA. Increasing dietary phenylalanine and tryptophan would increase the availability of precursor to the brain and hence restore the concentrations of the corresponding neurotransmitters.

A second possible explanation of the observed changes in neurotransmitter levels is that the reduction in food intake which occurs on feeding increased levels of the BCAA mixture is itself the cause of a reduction in the availability of the relevant amino acids to the brain for neurotransmitter synthesis. Supplementation with extra phenylalanine and tryptophan would then compensate for the reduced intake and hence restore concentrations to control values.

Each possible explanation does allow a combined supplement of phenylalanine and tryptophan to be of greater benefit than either added singly. The latter explanation would be supported by the positive correlations of brain NE, DA and 5HT concentrations with the levels of intake of food, protein and AME(N) and that of brain NE with intake of tyrosine+phenylalanine. However, slightly higher, though occasionally less significant, positive correlations are also apparent between the brain concentrations of NE and DA and the ratio of the dietary content of tyrosine+phenylalanine to that of the LNAA competing with them for brain uptake. This would tend to support the existence of competition

for brain uptake as proposed in the original explanation, particularly as the DA concentration was not correlated with the actual intake of tyrosine+phenylalanine. In addition, the brain concentration of 5HT was not correlated with the actual intake of tryptophan, but was correlated with the ratio of the dietary tryptophan content to that of the LNAA proposed to compete with it for uptake into the brain.

The pair-feeding of chicks fed the basal diet with those receiving that containing the highest BCAA supplement might perhaps aid clarification of the situation. It is apparent however that dietary variations in amino acid concentrations are here significantly influencing the levels of NE, DA and 5HT in the chick brain.

There is no evidence here to link the differences in brain neurotransmitter levels with the observed differences in growth of chicks fed the various diets, particularly as when neurotransmitter concentrations are restored to those of the basal diet by addition of phenylalanine and tryptophan to that diet having the highest BCAA content, growth is not fully restored and food intake remains depressed. The mechanism by which alterations in chick growth and food intake are caused by changes in the relative proportions of these dietary amino acids remains unclear.

4.12 Experiment 11. Excessive dietary content of branched-chain amino acids and increased supplementation with phenylalanine and tryptophan

a. Dietary nitrogen and AME(N) content (Table 29)

As in the previous investigation, it does not appear that the determined differences in dietary nitrogen content contribute greatly to the observed differences in chick growth and food intake, the control diet supporting the greatest chick growth and food intake while having the lowest nitrogen content. While the greater food intake of birds fed

the control diet might be attributed to its lower AME(N) content, their correspondingly greater growth seems unlikely to be directly due to this.

b. Growth and food intake (Figs. 33 and 34)

It is apparent that as in the previous experiment, phenylalanine supplementation is of some benefit in alleviating the effects of excessive incorporation of BCAA into the diet. However, even at the increased level of supplementation and length of feeding introduced here the effect is not consistently significant.

Data obtained from the previous experiment had indicated that a combined supplement of phenylalanine and tryptophan gave a greater improvement in growth and intake of chicks fed excessive levels of BCAA than either supplement given singly. In this experiment with increased amounts of both phenylalanine and tryptophan, no greater growth or intake is supported than by the phenylalanine supplement alone. It may be that the failure of additional tryptophan to reinforce the effect of phenylalanine in this experiment is due to the concentration of tryptophan employed. At a level of supplementation of 8g/kg the amino acid may be reaching levels in the diet at which it begins itself to exert a mildly toxic effect and thus nullifies any beneficial action which might be exerted in alleviating the adverse effects of the excessive dietary content of the BCAA.

c. Neurotransmitter concentrations (Table 30)

i. Effects of diet and food intake

After six days of feeding, the greater brain concentrations of 5HT and 5HIAA in chicks receiving the control diet or that incorporating the BCAA mixture, tryptophan and phenylalanine, compared with that of those fed diets containing the BCAA mixture alone or with

additional phenylalanine, is consistent with the existence of competition between these LNAA for uptake across the blood-brain barrier (Fernstrom, Larin and Wurtman, 1973; Olendorf and Szabo, 1976). An increase in the concentrations of the BCAA or phenylalanine would reduce the availability of tryptophan to the brain for neurotransmitter synthesis and hence brain concentrations of 5HT and its metabolite. Supplementation of the diet with tryptophan would increase its competition with the other LNAA for brain uptake and restore 5HT concentrations in the brain. The lower brain concentration of 5HIAA in chicks fed the diet containing the BCAA mixture and additional phenylalanine in comparison with those fed that incorporating only the BCAA mixture would also be explained by such a mechanism. The existence of these competitive effects is supported by the observation that the brain concentration of 5HT was not correlated with the actual quantity of tryptophan ingested, but was quite well correlated with the ratio of the dietary content of tryptophan to that of the LNAA proposed to compete with it for uptake into the brain. However, no similar effect was found for brain NE concentrations, these being correlated with the actual amount of tyrosine+phenylalanine ingested and not with the ratio of the dietary content of these amino acids to the LNAA possibly competing with them for brain uptake. Evidence for the functioning of a blood-brain barrier in the chick at this age thus appears to be conflicting.

Chicks fed the diet containing both the BCAA mixture and additional phenylalanine for fourteen days showed a higher brain NE concentration than those receiving the diet incorporating the BCAA mixture alone, indicating that this neurotransmitter is able to be influenced by increasing the dietary concentration of one of its

precursor amino acids. This is supported by the positive correlation of brain NE concentrations with the quantity of tyrosine+phenylalanine consumed. The rather weaker correlation with the level of protein intake which was also observed may result from the former effect.

An decreased turnover of DA in chicks receiving the diet containing only the BCAA is suggested by their reduced brain concentration of HVA in comparison with those fed the control diet. As at the sixth day of the feeding period, higher brain concentrations of 5HT in chicks fed the control diet or that containing the BCAA mixture and supplemented with both tryptophan and phenylalanine than in those fed the other diets, support the existence of a functional blood-brain barrier in chicks of this age. It is also apparent that the dietary concentration of tryptophan is influential in determining the brain concentration of 5HT. The greater brain 5HIAA concentrations in birds fed the control diet or that incorporating the BCAA mixture with tryptophan and phenylalanine than in those fed the diet supplemented with phenylalanine alone, also indicates that dietary tryptophan may affect brain 5HT metabolism. Again, the existence of competition between the LNAA for brain uptake is supported by the positive correlation of brain 5HT concentrations with the ratio of the dietary content of tryptophan to that of the LNAA likely to compete with it for brain uptake, and not with the actual amount of tryptophan consumed.

The maintenance until the end of the feeding experiment, of the difference in brain NE concentrations between chicks fed the diet incorporating the BCAA mixture alone and those receiving the BCAA mixture with additional phenylalanine, strengthens the evidence that this amino acid at the concentrations employed here does affect brain NE concentrations. Once more, this is supported by the positive correlation

of brain NE concentrations with the quantity of tyrosine+phenylalanine consumed. The reason for the loss in significance of the differences in brain 5HT concentrations at this point is unclear, no correlation being found between these and the ratio of tryptophan intake to that of its apparent competitors for brain uptake. The weak positive correlation of brain 5HT concentrations with the level of protein intake is not readily explained and seems insufficient to suggest a direct effect of the former on the latter, but is consistent with the report by Ashley and Anderson (1975b) that a reduction in the brain concentration of 5HT in the rat was accompanied by a fall in protein intake. These observations however, were made on rats which were allowed to select their protein and carbohydrate separately, and had their brain concentrations of 5HT depleted by administration of pCPA. Other workers have found no correlation of brain concentrations of 5HT with the level of protein intake (Peters and Harper, 1983).

It is apparent that, as in the previous investigation, the brain concentrations of NE, DA and 5HT may be affected by alterations in the dietary concentrations of their precursors. Some evidence is provided for the existence of competition between the LNAA for brain uptake. No information has been obtained which would imply a direct relationship between the brain neurotransmitter concentrations and growth of the birds fed the different diets.

ii. Effects of age and length of feeding period

After fourteen days of feeding, the transient fall in brain NE concentrations in chicks fed the control diet or that incorporating only the BCAA mixture is not observed in birds fed either of the diets which contain a phenylalanine supplement. This may be due to some alteration in the turnover of this neurotransmitter in chicks which have

a greater amount of the precursor amino acid available in the diet. The increase in the brain concentration of HVA in birds which had been fed the diet incorporating the BCAA mixture alone for the entire experimental period appears to indicate an increase in their breakdown of NE. The transient rise in brain HVA concentrations after fourteen days of the feeding period, shown in chicks consuming the diet containing both the BCAA mixture and a phenylalanine supplement, would tend to support such an effect of BCAA incorporation. In addition, the brain concentrations of 5HIAA in birds receiving diets containing the BCAA mixture alone or with only phenylalanine showed some increase with age and the length of the feeding period. This is consistent with an increase in turnover of 5HT, the effect apparently being removed by a supplement of tryptophan in addition to that of phenylalanine. However, no fall in the actual brain concentrations of 5HT with time is observed, and a possible reason for a supplement of the neurotransmitter precursor causing a reduction in the breakdown of 5HT is not apparent.

The brain neurotransmitter concentrations of chicks fed diets in this investigation which were identical to those employed in the previous study do show some differences at the fourteenth day of feeding. The growth and food intake of chicks fed the diet incorporating the maximum concentration of the BCAA mixture tended to be lower in this investigation than in that previously conducted. This is probably attributable to the chicks utilised in the more recent study being approximately 10g lighter at an age of seven days when experimental diets were introduced than those originally investigated. The apparently lower brain concentrations of some of the neurotransmitters and metabolites measured in chicks of the current investigation compared with those previously studied can only be attributed to variations in

the particular chicks employed and may again be an effect of their weight and possibly state of development at the introduction of the experimental diets.

4.13 Experiment 12. The effect of additional tryptophan on chicks fed high concentrations of dietary phenylalanine

a. Dietary nitrogen and AME(N) content (Table 31)

The observed differences in growth and food intake of chicks receiving the experimental diets do not appear to be attributable to the differences in nitrogen or AME(N) content, there being no consistent variation in either of these measurements with either the growth or the food intake of birds fed the experimental diets.

b. Growth and food intake (Figs. 35 and 36)

It is apparent that incorporation of phenylalanine into the diet at a concentration of 20g/kg results in a depression in chick growth and food intake which is alleviated by concomitant addition of tryptophan, the higher concentration of the latter amino acid resulting in the best chick growth. At an incorporated concentration of 40g/kg the effect of phenylalanine is more severe. While additional tryptophan at is of some benefit, the higher supplement possibly being the more effective, chick performance remains depressed relative to that of birds fed the control diet.

Thus the report by Elkin and Rogler (1983) of the possible antagonism of tryptophan by a dietary excess of phenylalanine in the chick has been confirmed. It is possible that a larger supplement of tryptophan might have been of greater benefit in alleviating the adverse effects of phenylalanine at an incorporated concentration of 40g/kg, nevertheless, the amounts added did result in growth and food intake of chicks consuming the diets being restored to that of birds fed that

containing half the quantity of phenylalanine in the absence of tryptophan supplementation.

c. Neurotransmitter concentrations (Table 32)

The lack of significant difference in the brain concentrations of NE and DA in chicks fed the various diets suggests that these neurotransmitter concentrations are not here influenced by the dietary intake of phenylalanine. This is also supported by the lack of correlation of brain NE or DA concentrations with the quantity of tyrosine+phenylalanine ingested and is also consistent with the report of Wurtman *et al.* (1974) that administration of phenylalanine did not increase catecholamine synthesis. Some indication of an increase in turnover of DA in chicks fed the diet incorporating the largest amounts of both phenylalanine and tryptophan is however given by their increased brain concentrations of DOPAC compared with birds receiving any of the other diets but that containing this quantity of phenylalanine and the smaller tryptophan supplement. In addition, chicks fed the diet supplemented only with tryptophan had a lower brain concentration of DOPAC than those reciving diets supplemented only with phenylalanine. Therefore it appears that the relative dietary concentrations of tryptophan and phenylalanine may have some effect upon the metabolism of DA.

An increased brain concentration of 5HT in chicks fed the diet supplemented only with tryptophan compared with those fed the control diets or that supplemented only with phenylalanine is consistent with alterations in tryptophan availability influencing the metabolism of this neurotransmitter (Fernstrom and Wurtman, 1971b). A comparable effect in chicks fed the diet containing phenylalanine at 20g/kg with tryptophan at 4g/kg compared with the same diets would support this, as

would the greater brain concentrations of 5HIAA in these birds than in those fed other diets. Other comparisons provide similar information. The positive correlation of the brain concentration of 5HT with the quantity of tryptophan consumed is again an indication that the former has some dependence on the latter. Accompanying positive correlations of the brain 5HT concentration with the amounts of food, protein and AME(N) ingested are weaker and, being somewhat interdependent, may merely be coincidental with the effect of tryptophan intake.

While in this investigation an increase in intake of dietary tryptophan has usually resulted in an increase in the brain concentration of 5HT, a continued rise in this concentration as dietary tryptophan intake is raised further is not apparent. Data indicates that an effect of tryptophan intake upon brain 5HT concentrations may be seen only within certain ranges of concentration of 5HT. In addition, there is little evidence of an effect of increased phenylalanine intake on brain concentrations of the catecholamines. The different observations on concentrations of catecholamines and 5HT in the chick brains possibly may be because the control diet was adequate in its content of tyrosine and phenylalanine but slightly deficient in tryptophan. It is perhaps therefore more likely that increases in the latter amino acid would affect brain 5HT concentrations than increases in the former would affect concentrations of the catecholamines.

4.14 Experiment 13. The effect of additional phenylalanine on chicks fed high concentrations of dietary tryptophan

a. Dietary nitrogen and AME(N) content (Table 33)

The variation in nitrogen content might be considered to have contributed to the response of the chick to the diets fed, the growth and food intake of the birds tending to increase with the dietary

nitrogen content. However, those fed excess tryptophan alone and with phenylalanine supplements showed little significant difference in their growth and food intake, despite some variation in the dietary nitrogen content. It is therefore unlikely that such variations have contributed greatly to the food intake and growth responses observed.

It is also apparent that the differences in dietary AME(N) content cannot account for the observed differences in chick performance, since no significant difference is observable between the AME(N) content of the diet supplemented with phenylalanine alone and that of the diet containing added tryptophan at a concentration of 30g/kg and phenylalanine at 20g/gk diet; yet these diets support very different levels of growth and food intake.

b. Growth and food intake (Figs. 35 and 36)

The control diet employed is obviously deficient in phenylalanine as expected, a supplement of this amino acid improving chick performance. Evidence for alleviation of the effects of excess tryptophan by phenylalanine is however fairly weak. At a level of 12g/kg, phenylalanine may be of some benefit in partially alleviating the effects of excess tryptophan and extending the feeding period might have resulted in the somewhat improved response of chicks receiving this supplement becoming significant, but at the higher level of phenylalanine supplementation it appears that a toxic effect due to increased dietary phenylalanine (as reported by Elkin and Rogler (1983) and in the previous investigation) has been introduced. It seems therefore that while the effects of a dietary excess of phenylalanine may be partially alleviated by additional tryptophan there is little of a reciprocal nature in this, that is, the effects of excessive dietary

tryptophan are not in turn alleviated to any significant extent by phenylalanine.

c. Neurotransmitter concentrations (Table 32)

The greater brain concentration of NE in chicks fed the diet incorporating both excess tryptophan and a supplement of phenylalanine at a concentration of 8g/kg than in those receiving the control diet is an indication that intake of phenylalanine may here influence the brain concentrations of NE. However, the failure of higher phenylalanine concentrations to have a similar effect and the lack of correlation of brain NE concentrations with the amounts of food, protein, AME(N) or tyrosine+phenylalanine ingested does not support such a suggestion. As birds consuming the control diet or that supplemented with phenylalanine alone at a concentration of 8g/kg had a lower brain concentration of DA than those fed any of the diets containing excess tryptophan alone or with varying amounts of phenylalanine, it appears that the addition of tryptophan may itself have played some role in raising the brain concentration of this neurotransmitter. The brain concentration of DA is negatively correlated with the quantity of food, protein and AME(N) consumed and not correlated with the total intake of tyrosine+phenylalanine. The effect thus is not due to differences in intake of the precursor amino acids. No possible competition between the LNAA for uptake across a blood-brain barrier would explain a tryptophan-caused increase in brain DA, in fact such competition ought to produce the opposite effect (Fernstrom, Larin and Wurtman, 1973). The cause of the observed increase remains to be elucidated.

Greater brain concentrations of 5HT in chicks fed diets containing excess tryptophan alone or with additional phenylalanine at concentrations of 8g/kg or 20g/kg are consistent with the brain

concentration of this neurotransmitter being dependent upon the availability of tryptophan in the diet. This again is supported by the positive correlation of brain 5HT concentrations with tryptophan intake. Accompanying correlations with the amounts of food, protein and AME(N) consumed are less strong and appear to be coincidental with the effect of tryptophan. The failure of the brain 5HT concentration of chicks receiving the diet incorporating excess tryptophan with a phenylalanine supplement of 12g/kg to reach significance compared with that of chicks fed diets lacking excess tryptophan is not readily explained. The measured concentration was however numerically higher, and not significantly less than that of those fed the diet containing excess tryptophan alone or supplemented with other concentrations of phenylalanine.

An increase in the brain concentration of 5HIAA in chicks fed the diet incorporating excess tryptophan alone or in combination with phenylalanine at 8g/kg, compared with that of birds fed other diets indicates that phenylalanine tends to reduce concentrations of this compound and suggests that there is a decreased breakdown of 5HT here. Such an effect might be due perhaps to a reduction in availability of tryptophan for its synthesis, as could result from a raised concentration of phenylalanine in the diet and hence the circulation causing an increase in competition with tryptophan for brain uptake.

The feeding of different amounts of phenylalanine and tryptophan has been shown here to affect the brain concentration of the neurotransmitters of which they are precursors. While chicks fed diets containing excess tryptophan and having the lowest levels of growth also had greater brain concentrations of 5HT, there is little to suggest any connection between the observable differences in growth of chicks

fed the different diets and the measured concentrations of the neurotransmitters.

4.15 General conclusions

Each of these studies has confirmed that variations in the amino acid balance of a diet can have quite severely detrimental effects on chick performance, consideration of all possible interactions between the various amino acids being necessary when formulating a diet in order to allow optimal growth. Information obtained from the above investigations indicates that in the young male broiler chick, brain concentrations of the catecholamines, 5HT and the measured metabolites may indeed be altered by changes in the amino acid content and balance of the diet presented.

An alteration in the brain concentrations of certain of the neurotransmitters and metabolites measured occurs with age -or length of the feeding period- under some of the dietary conditions imposed. These alterations do not appear to follow a pattern, such as a reduction in concentration with age, which might be attributable to an ongoing maturation of the blood brain barrier as suggested by Levi and Morisi (1971) and Purdy and Bondy (1976). As Pscheidt and Tamimie (1966) have indicated that the brain concentrations of NE and 5HT in the chick do not change after the first week of life, it may be that the alterations in neurotransmitter and metabolite concentrations occuring with time are a consequence of the length of the feeding period rather than changes in the chick due to increased age.

It appears that the brain concentration of 5HT may indeed be increased in the chick by the increased consumption of tryptophan, this being consistent with the rise in brain 5HT concentrations reported by Fernstrom and Wurtman (1971b) in the rat after the intraperitoneal

injection of tryptophan. The report by Arimanana *et al.* (1984) that the administration of a tryptophan load to rats resulted in an increase in the brain concentration of tryptophan but not 5HT or 5HIAA conflicts somewhat with this and cannot readily be explained. However, the absence of a consistently good correlation of brain 5HT concentrations with tryptophan intake in the current investigations perhaps indicates that factors other than tryptophan availability to the brain are involved in the regulation of 5HT synthesis.

A change in 5HT concentrations in the current studies is commonly accompanied by a parallel change in the brain concentration of 5HIAA, implying that the breakdown of 5HT alters in an attempt to maintain brain concentrations of this neurotransmitter within a particular range. This would be supported by recent findings that an increase in 5HT synthesis in synaptosomes due to increased tryptophan availability was paralleled by an accompanying catabolism to 5HIAA (Wolf and Kuhn, 1986).

Similar effects of dietary phenylalanine and tyrosine on brain concentrations of NE and DA are apparent. An increase in dietary phenylalanine may indeed result in an increased brain concentration of these catecholamines, comparable to the effect of tyrosine reported in the rat by Gibson and Wurtman (1977, 1978). It appears that such an increase may not directly correlate with the amount of tyrosine+phenylalanine consumed. The effect of phenylalanine on brain catecholamine concentrations contrasts somewhat with reports that while the administration of tyrosine to the rat would increase catecholamine synthesis (Gibson and Wurtman, 1977), that of phenylalanine would have no such effect (Wurtman *et al.*, 1974). The reason for this previously-reported failure of phenylalanine to affect brain catecholamine synthesis

may be due to the shorter time period of administration of the amino acid -it being injected intraperitoneally- since its conversion to tyrosine in the brain or other tissues, particularly the liver, would first be required. In addition, any increase in tyrosine production within the brain might initially only compensate for a reduction in the brain uptake of this amino acid due to the competitive effect of additional phenylalanine. A greater length of time then would be required for an increase in brain tyrosine sufficient to influence catecholamine synthesis to be acheived.

Some evidence is provided for the existence of a functional blood-brain barrier in the chick in the first 4-5 weeks of life, although conflicting data have also been obtained. It should first be noted that in these investigations, consideration of competitive effects influencing the availability of the catecholamine precursors to the brain has been made with respect to the ratio of the dietary content of both tyrosine and phenylalanine to that of the other LNAA (tryptophan, leucine, isoleucine and valine) expected to provide competition for uptake. Other reports have considered only tyrosine as a precursor of the catecholamines and included phenylalanine as a competitor (eg. Fernstrom, Hirsch and Faller, 1976), but as the latter amino acid may act as a tyrosine source this action may be questioned. However, as the calculations performed in other cases involved plasma concentrations of the LNAA, the conversion of phenylalanine to tyrosine in the liver might be assumed to occur to such a degree that any further contribution of phenylalanine to tyrosine synthesis in the brain itself may be disregarded. For the calculation of dietary ratios employed in these studies, the treatment of phenylalanine as a tyrosine source seems more fitting.

The degree to which the dietary neurotransmitter precursor:LNAA ratios are reflected in their actual plasma ratios is not absolutely certain, since a dietary excess of one amino acid may result in the specific depletion of the plasma concentration of another (eg. D'Mello and Lewis, 1970; Tews and Harper, 1982). However, data from the experiments are at several points consistent with what might be expected from competition between the LNAA for uptake into the brain. This is particularly so with respect to the observed effects of supplementation of diets containing varying concentrations of the BCAA with tryptophan and phenylalanine. Conflicting data is also available however, which would be consistent with the immaturity of the blood brain barrier in the young chick as suggested by Levi and Morisi (1971) and Purdy and Bondy (1976). No definite conclusion is able to be drawn regarding the possible state of development of such a barrier and accompanying transport mechanism, although the failure of Pscheidt and Tamimie (1966) to find changes in the brain concentration of 5HT and NE in the chick after the first week of life might indicate that the young chick brain is more mature than has been proposed.

It has been shown in the rat that while a correlation between the plasma tryptophan:LNAA concentration ratio and the brain concentration of 5HT exists, it is fairly weak over the normal dietary range (Arimanana *et. al.*, 1984). It is perhaps therefore not surprising that the experimental data obtained in these investigations do not consistently indicate an influence of the competitive effects of LNAA on chick brain neurotransmitter concentrations. Nevertheless, an interaction between the BCAA and phenylalanine and tryptophan-the precursors of NE, DA and 5HT-has been demonstrated in the chick, both in terms of effects on growth and food intake and also the brain concentrations of these

neurotransmitters.

There is no real evidence supplied here of the observed differences in brain concentrations of any of the neurotransmitters measured being a cause of the differences in intake of the various diets. Data from effects obtained from the direct injection of certain of the neurotransmitters and their depletion during the production of hypothalamic lesions affecting food intake, would indicate that these compounds do have some role to play in this phenomenon. However, while the addition of phenylalanine or tryptophan may restore the food intake and growth of chicks receiving diets which are deficient in that particular amino acid, and also increase the brain concentrations of the neurotransmitters of which they are the precursors, the two effects do not neccessarily accompany each other. In several of these investigations chick food intake and growth has been depressed without any consistent change in the brain concentrations of any of the measured neurotransmitters. While there is a suggestion that the brain concentrations of NE, DA and 5HT are not proportionally dependent on the dietary concentration of their precursors, it is not possible to completely separate changes in food intake and brain neurotransmitter concentrations into a cause and an effect. Altered food intake may indeed influence brain concentrations of these neurotransmitters, but whether these in turn may bring about a change in the food intake is not at all clear. Most observed correlations of neurotransmitter concentrations with the amounts of food, protein or AME(N) consumed are coincidental with correlations with the levels of intake of their precursor amino acids and as such cannot necessarily be assumed to be effects in their own right.

4.15 Future Work

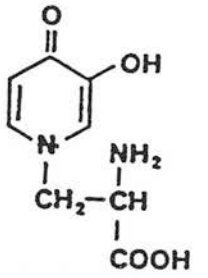
Further work on the subject of dietary amino acid imbalance and its effect on the brain concentrations of the neurotransmitters measured here is required in order to elucidate the mechanisms by which the effects observed are brought about. In the investigations described here, a considerable degree of variation in brain concentrations of the measured neurotransmitters was apparent between chicks fed the same diet. This might have been due to the birds consuming food at different times before killing. Overnight fasting in order to avoid this effect however, could have removed any diet-induced differences in brain concentrations of the compounds studied. It is possible that the training of the chicks to consume a diet within a particular time period might be of benefit in reducing differences observed between birds fed the same diet. In addition, the use of a larger number of chicks in feeding experiments could aid clarification of the trends occurring.

Studies on chicks allowed to choose between different diets would also be of interest. In particular, allowing chicks simultaneous access to a diet imbalanced, that is, relatively deficient in one amino acid, and another containing an excess of the same amino acid might provide some insight into the mechanisms regulating the intake of food, since choosing an appropriate proportion of each would give the chick a well-balanced diet. The pairfeeding of birds in order to equalise the intake of control diets and those causing a depression in food intake and growth might also aid differentiation between effects due to differences in the amount of food consumed and those caused by differences in the dietary formulation.

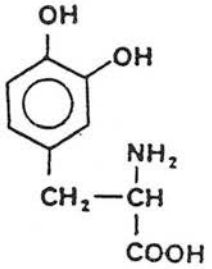
The possible influence of the total dietary amino acid content on the brain concentrations of the neurotransmitters measured

might further be investigated by a determination of the effects of an excessive amount of one amino acid precursor, e.g. tryptophan, as the total dietary protein content was progressively increased. The effects of maintaining a constant tryptophan content or a constant relative excess with increasing dietary protein would perhaps merit comparison. However, as the brain concentration of 5HT at least, has been suggested to vary with changes in the proportion of protein to carbohydrate in the diet (Fernstrom and Wurtman, 1974), it may be necessary to consider such possibilities when evaluating the data obtained.

Of more immediate applicability to the feeding and growth of poultry is the study of materials currently employed or under investigation for use as feedstuffs, which could provide a great deal of information, particularly in the case of tropical products such as the legume *Leucaena Leucocephala*. This is known to contain an amino acid having a detrimental effect on food intake and growth. This amino acid, mimosine ((S)-β-[N-(3-hydroxy-4-pyridone)]-α-aminopropanoic acid) is structurally related to DOPA (Fig 39). This hydroxylation product of tyrosine is the immediate precursor of DA and therefore also a precursor of NE. An evaluation of Leucaena leaf meal as a potential source of protein for the young chick has been made by Acamovic (1987). That the adverse effects of feeding such material may be due in part to some effect of mimosine on the biosynthesis or action of the catecholamines is a possibility that warrants further investigation. In the study and development of novel feedstuffs, the investigation of the balance and content of amino acids in the diet and consequent effects on brain neurotransmitter concentrations and chick performance must be of considerable interest. When the metabolic consequences of feeding diets which are distorted in their amino acid balance are clarified, it may be



Mimosine



3,4-dihydroxyphenylanine (DOPA)

Fig. 39. The structural relationship of mimosine and DOPA

possible to develop methods of preventing the adverse effects of amino acid interactions during the practical feeding of poultry.

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APPENDIX

Table A1. Composition of minerals+choline mixture employed in all experiments

COMPOUND	AMOUNT ADDED(kg)
$CaHPO_4.2H_2O$	5.556
$CaCO_3$ (Limestone flour)	3.591
NaCl	0.681
$MnSO_4.H_2O$	0.0494
$Zn(CH_3COO)_2.2H_2O$	0.03852
$FeSO_4.7H_2O$	0.02284
$Na_2MoO_4.2H_2O$	0.001157
$Na_2SeO_4.10H_2O$	0.107×10^{-3}
$Co(CH_3COO)_2.4H_2O$	0.096×10^{-3}
KI	0.30×10^{-3}
$CuSO_4.5H_2O$	1.802×10^{-3}
Choline chloride	0.2294
	10.172

Table A2. Composition of vitamin mixture added to all diets

COMPOUND	AMOUNT ADDED (g)
RETINOL	14.02
α-TOCOPHEROL	13.92
RIBOFLAVIN	4.67
CHOLECALCIFEROL	3.50
MENADIONE.$NaHSO_4$	1.1682
CYANOCOBALAMIN	0.0116
NICOTINIC ACID	23.36
CALCIUM PANTOTHENATE	11.684
FOLIC ACID	2.336
VIRGINIAMYCIN (2%)	292.1
BUTYLATED HYDROXY-TOLUENE	146.0
COCCIDIOSTAT	146.0
'ADDITIONAL VITAMINS'*	23.32
	682.0898

*See Table A3

Table A3. Composition of 'additional vitamins' added to vitamin mixture

COMPOUND	AMOUNT ADDED(g)
THIAMINE.HCl	23.97
PYRIDOXINE.HCl	1.44
d-BIOTIN	0.145
i-INOSITOL	23.97
p-AMINOBENZOIC ACID	0.48
	50.005

Table A4. Analysis of variance for an experiment of Latin square design.

Nature of observations:-
Concentrations of NE in the chick brain after six days of feeding an excessive amount of a branched-chain amino acid mixture alone and with increased concentrations of tryptophan and phenylalanine (Experiment 11)

No. of diets, n=4 Total no. of observations, N=16
Diet totals=D Column totals=C Row totals=R
Individual values=y

Layout plan

Column numbers				Row No	Row Totals
1	2	3	4		
285	306	184	321	1	1096
237	236	257	295	2	1025
248	255	261	185	3	949
282	215	258	292	4	1047
1052	1012	960	1093		4117

Column totals

Diet table

	Diet numbers			
	I	II	III	IV
	285	184	321	306
	257	237	236	295
	255	185	261	248
	292	215	282	258
Totals:	1089	821	1100	1107
Means:	272	205	275	277

Sums of squares

Rows $\Sigma(R^2/n)-(\Sigma y)^2/N = 2807.19$

Columns $\Sigma(C^2/n)-(\Sigma y)^2/N = 2418.69$

Diets $\Sigma(D^2/n)-(\Sigma y)^2/N = 14497.19$

Total $\Sigma(y^2)-(\Sigma y)^2/N) = 23793.44$

Analysis of variance

	Sum of squares	d.f.	Mean square	Variance ratio
Rows	2807.19	3	935.73	1.38
Columns	2418.69	3	806.23	1.19
Diets	14497.19	3	4832.40	7.12***
Residual	4070.37	6	678.39	
Total	23793.44	15		

*P<0.05, **P<0.01, ***P<0.001

Standard error of a difference between two diet means

= 2xresidual mean square/n = 18.42

Least significant differences between two diet means :-

$t_{(0.05,6)}$x18.42 = 45 (P<0.05)
$t_{(0.01,6)}$x18.42 = 68 (P<0.01)
$t_{(0.001,6)}$x18.42 = 110 (P<0.001)

e.g. Mean brain NE concentrations of birds fed diets II and IV are significantly different (P<0.01)